N-NITROSAMINES IN DRY FERMENTED SAUSAGES: OCCURRENCE AND FORMATION OF *N*-NITROSOPIPERIDINE

Eveline DE MEY

Supervisors:
Prof. G. Derdelinckx, KU Leuven
Prof. H. Paelinck, KU Leuven@KAHO Sint-Lieven

Members of the Examination Committee:
Prof. J. Buyse, KU Leuven, chair
Dr. I. Fraeye, KU Leuven@KAHO Sint-Lieven
Prof. M.-C. Peeters, KU Leuven
Prof. B. Goddeeris, KU Leuven
Prof. S. De Smet, UGent
Prof. Y. Vander Heyden, VUB
Prof. T. Kowalska, University of Silesia, Poland

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VOORWOORD

Ik ben trots dat ik u dit werk kan voorleggen. Niet zozeer, dit proefschrift als object op zich. Maar als tastbaar resultaat van een periode met uitdagingen kennisverwerving en heel veel doorzettingsvermogen. Ik moet toegeven dat dit niet de eenvoudigste periode in mijn leven is geweest, maar bij nader inzien wens ik dat het nooit eindigt. Het is verslavend om steeds op zoek te gaan naar de grenzen van je eigen (intellectuele) kunnen. En in mijn ogen is dit dan ook het doel van doctoraal onderzoek; gebeten worden door die academische microbe, voor elke 'waarom' een gefundeerd antwoord willen vinden en daarbij op tien nieuwe 'waarommetjes' stuiten.

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[1] Note: "The document reflects the author's views. The interreg IVA 2 Seas Programme Authorities are not liable for any use that may be made of the information contained therein."

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Eveline,
Gent, Februari 2014

SAMENVATTING

Het voorkomen van kankerverwekkende stoffen zoals *N*-nitrosamines in voeding is een niet te negeren voedselveiligheidsprobleem. Aangezien de consumptie van voeding gecontamineerd met *N*-nitrosamines kan leiden tot de vorming van kankercellen in verscheidene weefsels, dient gestreefd te worden naar een totale afwezigheid van deze contaminanten. Ook in vleeswaren worden *N*-nitrosamines teruggevonden, zij het meestal in lage concentraties. Algemeen wordt aangenomen dat *N*-nitrosamines gevormd worden door de nitrosylering van een secundair amine met een nitrosylerend agens. In droge gefermenteerde worst is het nitrosylerend agens hoofdzakelijk afkomstig van het gebruik van natriumnitriet, dat fungeert als conserverend en kleurvormend agens. Daarnaast kunnen micro-organismen ervoor zorgen dat er tijdens de fermentatie en de daaropvolgende rijping van de worst aminozuren gedecarboxyleerd worden tot biogene amines. Deze biogene amines, waarvan sommigen op zich voedselvergiftigingen kunnen veroorzaken, moeten op hun beurt omgezet worden tot secondaire amines om directe precursoren te zijn van *N*-nitrosamines. Biogene amines worden daarom ook beschouwd als een risico voor de vorming van *N*-nitrosamines in droge gefermenteerde worst. Deze hypothese is echter nog niet experimenteel bewezen. Dit werk heeft dan ook als doel extra inzicht te verwerven in de aanwezigheid en de vorming van vluchtige *N*-nitrosamines, in relatie tot de beschikbaarheid van nitriet en de accumulatie van biogene amines als mogelijke precursoren. In het bijzonder werd de focus gelegd op het vormingsmechanisme van *N*-nitrosopiperidine (NPIP) in droge gefermenteerde worst model.

In eerste instantie was het noodzakelijk een HPLC methode te ontwikkelen voor de bepaling van biogene amines in droge gefermenteerde worst. Om de detectiegevoeligheid te verhogen was een derivatisatie noodzakelijk. Hierbij werd de veelgebruikte dansylatie vergeleken met een alternatieve dabsylatie procedure. De derivatisatie met behulp van dabsyl chloride kreeg de voorkeur aangezien deze procedure uitgevoerd werd bij 70°C gedurende 25 min i.p.v. bij 40°C gedurende 45 min, wat een tijdswinst betekende. De interferenties in het chromatogram, afkomstig van de complexe eiwit-vet matrix, konden vermeden worden door ook een vastefase-extractie (SPE) uit te voeren. Hierdoor werd een betrouwbare en gevoelige methode bekomen om de biogene amines, namelijk tryptamine (TRYP), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonine (SER), tyramine (TYR), en de natuurlijke polyamines spermidine (SPD) en spermine (SPM), te bepalen in droge gefermenteerde worst.

Vervolgens werden de concentraties aan *N*-nitrosamines, biogene amines, en residueel nitriet bepaald in 101 commerciële droge gefermenteerde worsten die verkrijgbaar zijn op de Belgische markt. Zo werd de voedselveiligheid van de huidige commerciële producten, in relatie tot de

aanwezigheid van deze componenten, geëvalueerd. Over het algemeen werd vastgesteld dat de producten veilig waren aangezien de concentraties aan biogene amines en *N*-nitrosamines laag bleven. Enkel lage concentraties aan *N*-nitrosomorpholine (NMOR) en NPIP werden in een redelijk hoog percentage aan stalen (resp. 22% en 28%) gedetecteerd. In 3% van de gevallen werden zelfs kwantificeerbare hoeveelheden NPIP (hoger dan de methode kwantificeringslimiet (MQL) van 2,5 µg/kg) vastgesteld. Daarnaast werd principale-componentenanalyse (PCA) en hiërarchische clusteranalyse (HCA) uitgevoerd om verbanden te zoeken tussen de aanwezigheid van biogene amines en *N*-nitrosamines, en de relatie met fysische en chemische kenmerken. Er kon echter geen correlatie gevonden worden tussen de *N*-nitrosamines contaminatie en de hoeveelheid biogene amines of de fysische en chemische kenmerken van de commerciële vleesproducten. Bovendien kon de *N*-nitrosamine contaminatie niet gelinkt worden aan een specifiek type droge gefermenteerde worst.

Om de *N*-nitrosamine vorming te bestuderen werd ervoor geopteerd te werken met een modelbereiding van droge gefermenteerde worst, geproduceerd onder strikt gecontroleerde omstandigheden (Good Manufacturing Practices, GMP). In het technicum van de Onderzoeksgroep voor Technologie en Kwaliteit van Dierlijke Producten werd een droge gefermenteerde worst, van het Noord-Europese type, ontwikkeld. De fysische (pH = 5,1 en a_w = 0.93) en chemische kenmerken (zout-, vocht-, vet- en eiwitgehalte waren respectievelijk 3,5; 42,9; 32,0 en 19,0 g/100g) kwamen grotendeels overeen met deze van commerciële producten. Bovendien werden tijdens de bereiding van dit droge gefermenteerde worstmodel geen extreme biogene amine accumulatie en geen *N*-nitrosaminevorming waargenomen. Hierdoor werd besloten dat dit model uitermate geschikt was als controlebereiding voor de verdere studie van de *N*-nitrosaminevorming.

In het tweede deel van het doctoraat werd de NPIP vorming meer in detail bestudeerd aangezien de commerciële stalen vooral gecontamineerd waren met NPIP.

Aan de hand van het worstmodel werd nagegaan wat de rol was van het biogeen amine CAD en de directe precursor piperidine (PIP) in de vorming van NPIP tijdens de productie van droge gefermenteerde worst. In deze studie werd de invloed van de pH (4,9 en 5,3), natriumnitriet (0 en 150 mg/kg) en natriumascorbaat (0 en 500 mg/kg) nagegaan. Wanneer de salamideeg verrijkt werd met een overmaat aan CAD (500 mg/kg cadaverine dihydrochloride, CAD.2HCl), werd er geen verhoogde NPIP vorming vastgesteld. In tegenstelling, de aanrijking met PIP (10 en 100 mg/kg), resulteerde in een verhoogde NPIP vorming. Enerzijds kon geen effect van het kleine pH verschil vastgesteld worden op de NPIP vorming. Anderzijds was de NPIP vorming meer uitgesproken wanneer de worst werd bereid met natriumnitriet en zonder natriumascorbaat. In het geval dat er een overmaat PIP aanwezig was, werd de rol van nitriet als bron van het nitrosylerend

agens en het belang van ascorbaat als *N*-nitrosamine inhibitor bevestigd. Dit effect werd echter enkel in het begin van de productie waargenomen aangezien NPIP tijdens de verdere productie degradeerde.

Vervolgens werd de invloed van de pH en a_w op de NPIP vorming bestudeerd aan de hand van proteïne-gebaseerde vloeibare systemen. In een eerste systeem (NaCl-systeem) werd de NaCl concentratie (0 - 30%) gevarieerd om de a_w te verlagen (tussen 0,99 en 0,79) bij twee verschillende pH's (4,0 en 5,0). Bij beide pH's werd vastgesteld dat de gevormde hoeveelheid NPIP daalde wanneer de a_w gereduceerd werd. De gebruikte zoutconcentraties in het NaCl-systeem, nodig voor de verlaging van a_w, waren echter veel hoger dan deze in droge gefermenteerde worst (ca. 3% NaCl). Hierdoor was het onduidelijk in welke mate dit effect toegeschreven kon worden aan de hogere ionsterkte van de oplossing of de verlaging van a_w. Daarom werd in een tweede systeem (PEG-systeem) polyethyleenglycol (PEG) gebruikt om de a_w te verlagen. Met behulp van respons oppervlak methodologie (RSM) werd de invloed van de pH (3,0 – 7,0), a_w (0,80 – 0,99) en de incubatie tijd (1,3 – 98,7 u) op de NPIP vorming nagegaan. Er werd vastgesteld dat de NPIP concentratie steeg bij een langere incubatietijd, hogere a_w en lagere pH. De resultaten, verkregen in de vloeibare systemen, droegen dus bij tot het verkrijgen van een beter inzicht in de inhibitie van de NPIP vorming tijdens de productie van droge gefermenteerde worst. Zo kan gesteld worden dat de mogelijke vorming van NPIP, deels bevorderd door de lichte pH-daling tijdens de fermentatie, geremd wordt door de verlaging van a_w.

Als laatste werd onderzocht of de aanwezigheid van NPIP in droge gefermenteerde worst afkomstig kan zijn van het gebruik van NPIP gecontamineerde kruidenmengsels. In eerste instantie werden analytische methodes ontwikkeld om de precursoren, namelijk piperine en PIP, te kunnen bepalen in kruiden. Piperine werd via HPLC-DAD (λ = 343 nm) bepaald na 'accelerated solvent extraction' (ASE) met dichloromethaan. Voor de bepaling van PIP via HPLC-ELSD werd eerst een hydroextractie uitgevoerd met behulp van ASE. De veelgebruikte kruiden in droge gefermenteerde worst, namelijk paprika (*Capsicum annuum*), chilipeper (*Capsicum frutescens*), piment (*Pimenta dioica*) en nootmuskaat (*Myristica fragrans*), bevatten enkel sporen van beide precursoren. Alleen in stalen van witte en zwarte peper konden grote hoeveelheden piperine (max. 21.12 mg/g) en PIP (max. 11.42 mg/g) gemeten worden. Niettemin, het gebruik van deze piperine en PIP bevattende kruiden in mengsels met nitrietpekelzout resulteerde niet altijd in de vorming van NPIP. Enkel in het mengsel van witte peper werd een kleine hoeveelheid NPIP (9.8 ng/g) teruggevonden na twee maanden bewaring. Deze hoeveelheid was echter niet voldoende om de sporadische aanwezigheid van kwantificeerbare concentraties aan NPIP in commerciële droge gefermenteerde worst te kunnen verklaren. Er dient hierbij dan ook opgemerkt te worden dat de bewaaromstandigheden optimaal waren en de periode relatief kort was.

Verder onderzoek kan uitwijzen of andere bewaaromstandigheden wel kan leiden tot sterk verhoogde concentraties van NPIP in kruidenmengsels.

De resultaten van dit werk tonen aan dat het risico op *N*-nitrosamines, meer bepaald NPIP vorming, in droge gefermenteerde worst, geproduceerd onder GMP condities, over het algemeen zeer klein is. Enerzijds werd bewezen dat biogene amines, toch zeker wat betreft CAD, geen aanleiding geven tot de vorming van NPIP tijdens de productie van droge gefermenteerde worst. Anderzijds kan NPIP enkel gevormd worden uit PIP indien extreme hoeveelheden PIP toegevoegd werden. Gebruikelijke hoeveelheden PIP, die geïntroduceerd kunnen worden in de worst via toevoeging van PIP bevattende kruiden, vormen hierbij geen risico op de vorming van NPIP. Het sporadisch voorkomen van kwantificeerbare concentraties aan NPIP in commerciële droge gefermenteerde worst zal hoogstwaarschijnlijk te wijten zijn aan het gebruik van NPIP gecontamineerde kruidenmengsels.

ABSTRACT

The occurrence of carcinogenic *N*-nitrosamines in food cannot be ignored as food safety issue. Since the intake of *N*-nitrosamine contaminated food may induce all kinds of cancer tumors, the presence of these carcinogens must be reduced to the lowest possible concentrations (below the limit of detection). Also in meat products *N*-nitrosamines can regularly be detected, although mostly in low concentrations. Generally, it is assumed that *N*-nitrosamines are formed by the nitrosation of a secondary amine with a nitrosating agent. In dry fermented sausages, the nitrosating agent mainly originates from sodium nitrite, which is added to the meat as preservative and colouring agent. In addition, during the fermentation and the subsequent ripening period, microorganisms can decarboxylate amino acid to biogenic amines. These biogenic amines, some of which can cause food poisoning themselves, may be transformed to the secondary amines, which are the direct precursors of *N*-nitrosamines. Therefore, biogenic amines are considered to be a risk for the formation of *N*-nitrosamines in dry fermented sausages. However, this hypothesis has not been confirmed experimentally yet. Therefore, the objective of this work was to gain additional insight in the occurrence and formation of *N*-nitrosamines in relation to nitrite and the accumulation of biogenic amines in dry fermented sausages. More specifically, the study was focused on the mechanism of *N*-nitrosopiperidine (NPIP) formation in a dry fermented sausage model.

Firstly, a method was optimised for the determination of biogenic amines in dry fermented sausages. To increase the sensitivity, a derivatisation was necessary. Therefore the commonly used dansylation was compared to an alternative dabsylation procedure. The derivatisation with dabsyl chloride was preferred since it was realized in 25 min at 70 °C instead of 45 min at 40 °C, which is a substantial time gain. The interferences in the chromatogram, which originated from the complex protein-fat matrix, were removed by a solid phase extraction (SPE). As a result, a reliable and sensitive method was developed to determine the biogenic amines, i.e., tryptamine (TRYP), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonin (SER), tyramine (TYR), and the natural polyamines spermidine (SPD) en spermine (SPM), in dry fermented sausages.

Next, the concentrations of *N*-nitrosamines, biogenic amines and residual nitrite were determined in 101 commercial dry fermented sausages, which were available on the Belgian market. In this way, the food safety of the commercial products in relation to the occurrence of these compounds were assessed. In general, the product could be considered safe since the concentrations of biogenic amines and *N*-nitrosamines remained low. Traces of *N*-nitrosomorpholine (NMOR) and NPIP were found in 22% and 28% of the samples, respectively. In some cases (3%), the NPIP concentrations were measured in quantifiable concentrations (above the method

quantification limit (MQL) of 2,5 µg/kg). In addition, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used in to search for patterns in the occurrence of biogenic amines and *N*-nitrosamines in the commercial meat products, and their relation with physical and chemical characteristics. However, no correlation could be observed between the *N*-nitrosamine contamination and the amounts of biogenic amines or the physical and chemical characteristics. Moreover, the contamination with *N*-nitrosamines could not be linked to a specific type of dry fermented sausage.

To study the *N*-nitrosamine formation, the use of a dry fermented sausage model, produced under strictly controlled conditions (Good Manufacturing Practices, GMP) was preferred. In the pilot infrastructure of the Research Group of Technology and Quality of Animal Products, a North European type dry fermented sausage was developed. The physical (pH = 5.1 and aw = 0.93) and chemical (salt, moisture, fat and protein content were 3.5, 42.9, 32.0, and 19.0 g/100g, respectively) characteristics were comparable with results found for commercial products. In addition, no extreme biogenic amine accumulation and no *N*-nitrosamine formation was observed during the production of the dry fermented sausage model. Therefore, this model was proven to be suitable for the study of the *N*-nitrosamine formation.

In the second part of this work, the NPIP formation was studied in detail since the commercial samples were mainly contaminated with NPIP.

By means of the dry fermented sausage model, the role of the biogenic amine CAD and the direct precursor piperidine (PIP) on the formation of NPIP was studied during the production of dry fermented sausages. In this study, the influence of pH (4.9 and 5.3), sodium nitrite (0 and 150 mg/kg) and sodium ascorbate (0 and 500 mg/kg) was investigated. When the meat batter was enriched with CAD (500 mg/kg cadaverine dihydrochloride, CAD.2HCl), no increased NPIP concentrations were observed. In contrast, the enrichment with PIP (10 and 100 mg/kg) resulted in an increased NPIP formation. On the one hand, no effect on the NPIP formation was seen by the small difference in pH. On the other hand, the NPIP formation was significantly higher when the sausages were prepared with sodium nitrite and when sodium ascorbate was omitted. In the case that PIP was present in excessive amounts, nitrite could be identified as source of the nitrosating agent. Also the role of ascorbate as inhibitor of *N*-nitrosamine formation was confirmed. However, this effect could only be observed in the beginning of the production since NPIP degraded during the subsequent production phases.

In the next part, the influence of pH and a_w on the NPIP formation was investigated, using two protein-based liquid systems. In the first system (NaCl-system), the NaCl concentration (0 – 30%) was varied to reduce the a_w (between 0.99 and 0.79) at two pH levels (4.0 and 5.0). At both pH levels, the reduction of a_w resulted in a decreased amount of NPIP. However, the applied NaCl concentrations, necessary for the reduction of a_w, were much

higher than the NaCl content in dry fermented sausages (ca. 3%). As a consequence, it was unclear to what extent this effect could be attributed to the higher ionic strength or the lower a_w. In a second system (PEG-system), poly ethylene glycol (PEG) was used to reduce the a_w. Response surface methodology (RSM) was applied to evaluate the combined effect of pH (3.0 – 7.0), a_w (0.80 – 0.99) and the incubation time (1.3 – 98.7 h) on the NPIP formation. It was observed that the NPIP concentrations increased when the incubation time was longer, the a_w was higher and the pH was lower. The results obtained in the liquid systems contributed to a better understanding of the inhibition of the NPIP formation during the production of dry fermented sausages. Hereby, the possible NPIP formation, partially promoted by a slight acidification during the fermentation, is inhibited by the reduction of a_w.

Finally, it was investigated if the presence of NPIP in dry fermented sausages can emanate from the use of NPIP contaminated spices. First, analytical methods were developed to determine the precursors, namely piperine and PIP, in the spices. Piperine was determined by HPLC-DAD (λ = 343 nm) after accelerated solvent extraction (ASE) with dichloromethane (DCM). To determine PIP, a hydroextraction by means of ASE followed by HPLC-ELSD was applied. Commonly used spices in dry fermented sausages, i.e., paprika (*Capsicum annuum*), chilli (*Capsicum frutescens*), allspice (*Pimenta dioica*), and nutmeg (*Myristica fragrans*), contained only traces of both precursors. Only in samples of black and white pepper high concentrations of piperine (max. 21.12 mg/g) and PIP (max. 11.42 mg/g) were measured. However, the addition of piperine and PIP containing spices in nitrite curing salt mixtures, did not always result in the formation of NPIP. Only in the mixture containing white pepper, a small amount of NPIP (9.8 ng/g) was detected after a two month storage period. However, this amount was not sufficient to explain the sporadic occurrence of quantifiable concentrations of NPIP in commercial dry fermented sausages. It should be noticed that the storage conditions were optimal and the storage time was relatively short. Future studies are needed to reveal if changed storage conditions can result in increased amounts of NPIP in the spice mixtures.

The results obtained in this work demonstrated that in general the risk of *N*-nitrosamine contamination in dry fermented sausages is low. On the one hand, it was proven that the accumulation of biogenic amines, especially with regard to CAD, will not result in the formation of NPIP during the production of dry fermented sausages. On the other hand, NPIP can be formed from PIP, but only when extreme concentrations of PIP are present. Common amounts of PIP, which can be introduced in the sausage by the addition of PIP containing spices, are not a risk for the formation of NPIP. The sporadic occurrence of quantifiable concentrations in commercial dry fermented sausages will probably be attributed to the use of highly NPIP contaminated spice mixtures.

LIST OF NOTATIONS

LIST OF ABBREVIATIONS

A	adenine
AGM	agmatine
ANOVA	analysis of variance
ASE	accelerated solvent extraction
atm	atmosphere
a_w	water activity
BHI	brain-heart-infusion broth
BPN	γ-butenyl(3-propenyl)nitrosamine
CAD	cadaverine
CCD	central composite design
DAD	diode array detector
DAO	diamine oxidase
DAOI	diamine oxidase inhibitor
Dbs-Cl	dabsyl chloride (4-dimethylaminoazobenzene-4-sulfonyl chloride)
DM	dry matter
DNA	deoxyribonucleic acid
Dns-Cl	dansyl chloride (1-dimethylamino-naphtalene-5-sulfonyl chloride)
Dns-N_2H_3	dansyl hydrazine
Dns-NH_2	dansylamide
Dns-OH	dansyl sulphonic acid
EC	European Commission
EC nr	Enzyme Commission number
ELSD	evaporative light scattering detector
EPIC	European Prospective Investigation into Cancer and Nutrition
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
G	guanine
GCC+	gram-positive coagulase positive cocci
GC-TEA	gas chromatography-thermal energy analyzer
GMP	Good Manufacturing Practices
HA	heterocyclic amine
HCA	hierarchical cluster analysis
HIS	histamine
HPLC	high performance liquid chromatography
IARC	International Agency for Research on Cancer
IS	internal standard
ISO	International Standard Organisation
LAB	lactic acid bacteria
LDC	lysine decarboxylase
LLE	liquid-liquid extraction
LOQ	limit of quantitation
LSE	liquid-solid extraction

MAO	mono amine oxidase
MAOI	mono amine oxidase inhibitor
MDL	method of detection limit
MGMT	O^6-methylguanine-DNA methyltransferase
MPR	moisture-protein ratio
MRS	de Man, Rogosa and Sharpe agar
MSA	mannitol salt agar
MSPD	matrix solid phase dispersion
MQL	method quantitation limit
NAB	*N*-nitrosoanabasine
NAT	*N*-nitrosoanatabine
NDBA	*N*-nitrosodibutylamine
NDBzA	*N*-nitrosodibenzylamine
NDEA	*N*-nitrosodiethylamine
NDMA	*N*-nitrosodimethylamine
NMOR	*N*-nitrosomorpholine
NNN	*N*-nitrosonornicotine
NOAEL	no-observable-adverse-effect-level
NPCA	*N*-nitrosopipecolic acid
NPIP	*N*-nitrosopiperidine
NPRO	*N*-nitrosoproline
NPYR	*N*-nitrosopyrrolidine
NTCA	*N*-nitrosothiazolidine-4-carboxylic acid
NTHZ	*N*-nitrosothiazolidine
ODC	ornithine decarboxylase
PAH	polycyclic aromatic hydrocarbon
PAO	poly amine oxidase
PC	principal component
PCA	principal component analysis
PDC	phenylalanine decarboxylase
PEG	poly ethylene glycol
PHE	phenylethylamine
PIP	piperidine
pKa	acid dissociation constant
PUT	putrescine
R^2	coefficient of determination
RCCD	rotatable central composite design
RH	relative humidity
RNA	ribonucleic acid
RP	reversed phase
RSD	relative standard deviation
RSM	response surface methodology
S/N	signal-to-noise ratio
SCX	strong cationic exchange cartridge
SER	serotonin
SPD	spermidine
SPE	solid phase extraction
SPM	spermine

T%	recovery
TCA	trichloro acetic acid
TDC	tyrosine decarboxylase
TEA	thermal energy detector
TMAO	trimethylamine *N*-oxide
t_R	retention time
TRYP	tryptamine
TYR	tyramine
USDA	United States Department of Agriculture
UV	ultraviolet
VRBG	violet red bile glucose agar
Y_{exp}	experimental value of the response
Y_{pred}	predicted value of the response

LIST OF USED CODES

CODES TO DESCRIBE VARIABLES FOR THE PCA IN CHAPTER 3

AN_AW	analysed water activity
AN_FAT	analysed free fat content
AN_NO_2	analysed residual sodium nitrite content
AN_NO_3	analysed residual sodium nitrate content
AN_pH	analysed pH
AN_SALT	analysed NaCl content
AN_WATER	analysed moisture content
AN-PROT	analysed protein content
CAL_MPR	calculated moisture-protein ratio
TOT_BA	total biogenic amine content
TOT_NA	total *N*-nitrosamine content

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GENERAL INTRODUCTION

N-Nitrosamine contamination is an important food safety issue, because most volatile *N*-nitrosamines are carcinogenic (IARC, 1978). In meat products, these carcinogens may be formed from available precursors, namely secondary amines and nitrite. The most frequently occurring *N*-nitrosamines in meat products are *N*-nitrosodimethylamine (NDMA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosomorpholine (NMOR).

In dry fermented sausages, these *N*-nitrosamines can also be found. For the production of dry fermented sausages, sodium nitrite is used. Although the use of nitrite forms an important source of nitrosating agents, the elimination of nitrite in meat products is almost impossible. The advantages of this additive are plural, most importantly it enhances the colour formation and inhibits the growth of the pathogen *Clostridium botulinum*. Therefore, the addition is not prohibited but restricted to a maximum of 150 mg $NaNO_2$/kg, (Directive 2006/52/EC, 2006). Moreover, the presence of biogenic amines in dry fermented sausages is considered to be an important source of nitrosatable amines. These compounds can accumulate during the fermentation and ripening of the sausage since microorganisms, either being introduced as starter culture or being present as contaminating flora, may decarboxylate free amino acids to biogenic amines. High concentrations of biogenic amines, mainly tyramine (TYR), putrescine (PUT) and cadaverine (CAD), may occur in the meat products. As a consequence, the presence of both precursors in dry fermented sausages constitutes a risk of *N*-nitrosamine formation.

More specifically, the occurrence of NPIP in dry fermented sausages is often related to the accumulation of CAD. However, this primary amine requires first a conversion to the alkaloid piperidine (PIP) prior to nitrosation. In a heated lean meat model, this conversion is achieved by intensive heating (Drabik-Markiewicz *et al.*, 2011). Interestingly, during the production of dry fermented sausages, no such high temperatures are used and the question arises if CAD can be designated as precursor in this kind of product. Besides the availability of CAD, PIP can also be introduced in the sausages by the addition of pepper. The main pungent compound of pepper, namely piperine, can be degraded to PIP (González-Mancebo, *et al.*, 2004). Thereby, PIP is omnipresent in sausages, nevertheless it does not necessarily result in the formation of NPIP. Probably, process conditions and product composition may influence the reaction mechanism.

The objective of this study is to gain additional insight into the occurrence and formation of *N*-nitrosamines in dry fermented sausages. This increased

knowledge may help to avoid any contamination of dry fermented meat products with these carcinogens. In particular, *NPIP* was studied because it is one of the most frequently detected *N*-nitrosamines in fermented meat products.

A schematic overview of the study is presented in Figure 1.

In **Chapter 1**, a literature review is given concerning the risk of *N*-nitrosamine contamination in meat products. Firstly, the chemical properties are described, including the carcinogenic character of the compounds. Secondly, an overview of the occurrence of *N*-nitrosamines in food stuff, especially in meat products, is given. Thirdly, current knowledge of the *N*-nitrosamine formation in meat products is discussed. Specific attention is paid to the critical factors related to their formation during the production of dry fermented sausages.

To study *N*-nitrosamine formation in dry fermented sausages, it is required to measure both the *N*-nitrosamines as well as their precursors, namely biogenic amines and nitrite. The analytical method for the determination of volatile *N*-nitrosamines can be acquired from the preceding PhD study of Drabik-Markiewicz (2010) and the determination of the nitrite and nitrate content can be based on methods found in literature. In addition, in **Chapter 2**, a method was developed to determine biogenic amines in dry fermented sausages.

In **Chapter 3**, a survey of dry fermented sausages of the Belgian market was performed. Of all the samples, the *N*-nitrosamines, biogenic amines and residual nitrite contents were determined together with a physical and chemical characterisation. In this way, the current *N*-nitrosamine contamination degree of dry fermented sausages was identified. In addition possible patterns in the occurrence of *N*-nitrosamines, biogenic amines, residual nitrite and nitrate and their relation to physical and chemical characteristics, was investigated.

Based on the results of Chapter 3, the work was focused on the formation of *NPIP*. In order to study the reaction pathway in a realistic, but controlled environment, a dry fermented sausage model was developed (**Chapter 4**). This model was then used in **Chapter 5** to study the formation of NPIP from the precursors CAD and PIP. Hereby, the influence of sodium nitrite and ascorbate on the NPIP formation during the production of dry fermented sausages was included.

Besides the availability of possible precursors, environmental conditions during the fermentation and ripening of the sausage can play an important role. In **Chapter 6**, a protein-based system was applied to investigate the influence of pH and water activity on the NPIP formation.

Finally, the possibility of external sources of precursors was investigated. In **Chapter 7**, a risk assessment of NPIP formation from PIP and piperine in

spices was performed. For that purpose, a method was developed to determine the piperine and PIP content in spices. As a proof of concept, the relation between the piperine and PIP content and the formation of NPIP was explored.

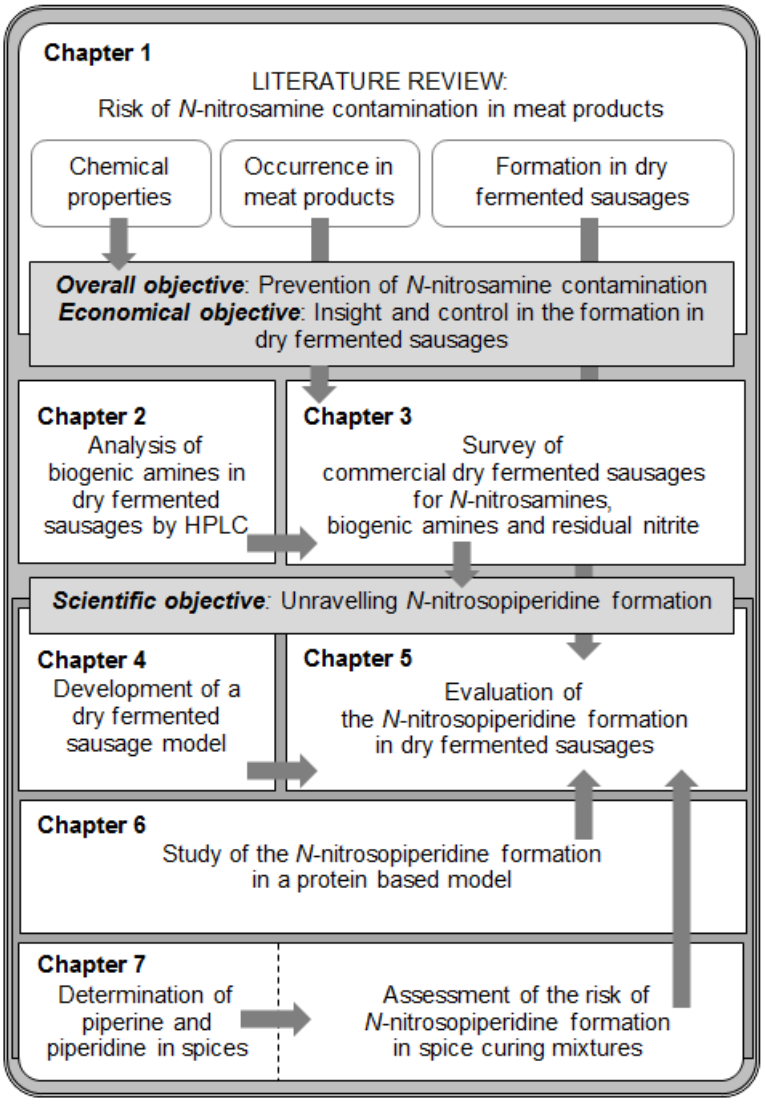


Figure 1 Schematic overview of the study.

CHAPTER 1
N-NITROSAMINE CONTAMINATION IN MEAT PRODUCTS: A LITERATURE REVIEW

1.1 INTRODUCTION

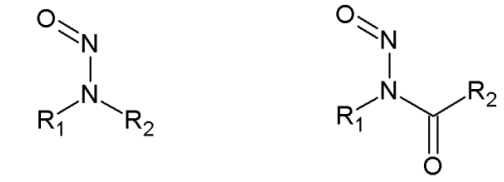
N-Nitrosamines are carcinogenic compounds which can occur in rubber, tobacco, cosmetics, soil, water, food products and many more. Since both precursors, namely nitrite and/or nitrate and (secondary) amines, are present in cured meat products, there is a potential risk of *N*-nitrosamines formation.

In the first chapter, a literature review is presented, consisting of three sections. In the first section, some general information about the classification, analysis and toxicology of *N*-nitrosamines is given. The second section presents an overview of the most common *N*-nitrosamines in food products, and shows insight in the formation of volatile *N*-nitrosamines in meat products. In the third section the risk of *N*-nitrosamine contamination in dry fermented sausage is discussed in detail, with focus on the ingredients/additives and meat processing parameters.

1.2 CHEMICAL PROPERTIES OF *N*-NITROSAMINES

1.2.1 CLASSIFICATION

N-Nitrosamines (R_1NNOR_2) are classified as a subgroup of the *N*-nitroso compounds, formed by the nitrosation of secondary amines. As can be seen in Figure 1.1, *N*-nitrosamines differ from the *N*-nitrosamides ($R_1NNOCOR_2$), by the lack of a carbonyl group attached to the nitrogen on which also the NO group is situated (Loeppky, 1994). In contrast to the unstable *N*-nitrosamides, attributed to the adjacent electropositive groups NNO and CO, *N*-nitrosamines are relatively stable over a broad pH range (Magee & Barnes, Carcinogenic nitroso compounds, 1967). Nevertheless they are light sensitive and undergo photo-oxidation during prolonged exposure to UV-light (Kodamatani *et al.*, 2009).

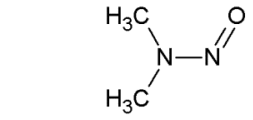


N-nitrosamine *N*-nitrosamide

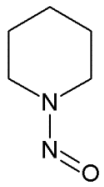
Figure 1.1 Chemical structure of *N*-nitrosamines and *N*-nitrosamides.

In nature, a wide diversity of secondary amines is present. Depending of the complexity of the amines, the resulting *N*-nitroso compounds can differ greatly in chemical structure and molecular weight. As a consequence, volatile and non-volatile *N*-nitrosamines can be identified based on their volatility in steam (Kubacki, 1979). *N*-Nitrosoamino acids, such as

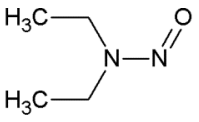
N-nitrosoproline (NPRO) and *N*-nitrosothiazolidine-4-carboxylic acid (NTCA), belonging to the group of non-volatile *N*-nitrosamines, show no carcinogenic properties (Tricker & Preussmann, 1991). In contrast, many volatile *N*-nitrosamines occurring in food are suspected to be human carcinogens. In Figure 1.2, the chemical structures of some food related *N*-nitrosamines are given.



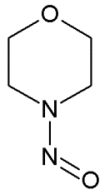
N-nitrosodimethylamine (NDMA)



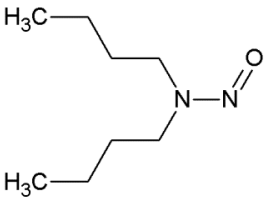
N-nitrosopiperidine (NPIP)



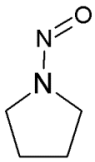
N-nitrosodiethylamine (NDEA)



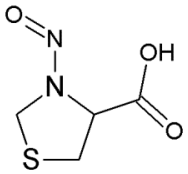
N-nitrosomorpholine (NMOR)



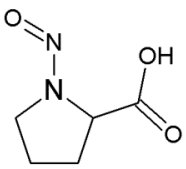
N-nitrosodibuthylamine (NDBA)



N-nitrosopyrrolidine (NPYR)



N-nitrosothiazolidine-4-carboxylic acid (NTCA)



N-nitrosoproline (NPRO)

Figure 1.2 Chemical structures of some *N*-nitrosamines.

1.2.2 ANALYSIS OF *N*-NITROSAMINES

The detection of the *N*-nitrosamines can be done by mass spectrometry (MS) (Wang, *et al.*, 2011; Grebel, Young, & Suffet, 2006) or by a Thermal Energy Analyzer (TEA) (Byun *et al.*, 2004). Although MS can be considered as the most eminent detector, the sample extract must be free of interfering compounds to avoid abundant background noise. Certainly, meat products, which are rich in proteins and fat, require an additional clean-up procedure (FSIS, 1991). In contrast, the TEA is highly selective for *N*-nitrosamines.

Although *N*-nitrosamines are considered to be relatively stable compounds, they typically show a weak N-NO bond. Based on this property, a highly selective and sensitive detector, namely the TEA detector was developed. The detection is based upon the thermal cleavage in the pyrolyzer of the weak N-NO bond of the *N*-nitrosamine, with release of a nitrosyl radical (•NO). In the reaction chamber, the nitrosyl radical is oxidized with ozone to obtain excited nitrogen dioxide (NO_2*). Subsequently, chemiluminescence is generated by the decay of the electronically excited NO_2* (Figure 1.3) (Fine *et al.*, 1975). Since only one NO for each *N*-nitroso group is generated, regardless of the presence of any other nitrogen groups, the detector shows equimolar response and allows reliable measurements of sub µg/kg quantities of *N*-nitrosamines in complex biological matrices (Yan, 2006).

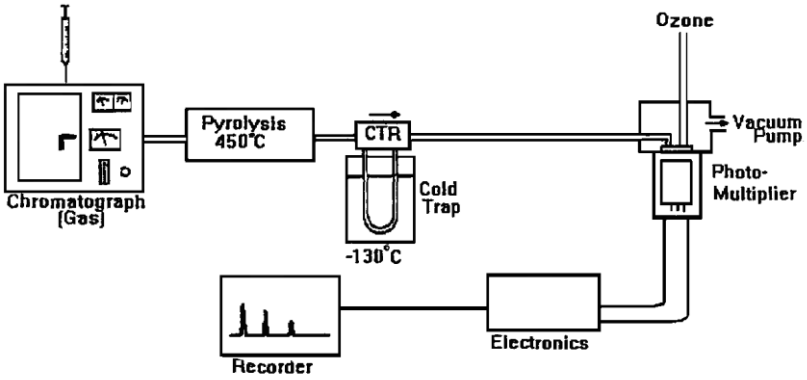


Figure 1.3 Diagram of the GC-TEA system (TEA ANALYZER model 610 Manual, n.d.)

Both volatile and non-volatile *N*-nitrosamines can be analysed by TEA. Although volatile *N*-nitrosamines are easily separated by gas chromatography (GC) prior to detection, the non-volatile compounds must firstly be derivatised by methylation or silylation (Kubacki, 1979). Also many attempts are made to couple HPLC to the TEA detector. However certain drawbacks, such as the necessity of using non-aqueous eluents (Sen & Kubacki, 1987), counter the research for the determination of individual non-volatile *N*-nitrosamines, *N*-nitrosoamino acids and *N*-nitrosamides.

1.2.3 CARCINOGENICITY AND EPIDEMIOLOGY

For a long time, *N*-nitrosamines were considered as a non–interesting group of chemical compounds until Magee and Barnes (1956) demonstrated hepatic carcinogenesis of NDMA during prolonged oral administration to rats. In the subsequent decennia, many studies followed on a wide variety of animals and of the more than 300 tested *N*-nitroso compounds, 90 % were found to have carcinogenic properties (Preussmann & Stewart, 1984). Moreover, most *N*-nitrosamines induce organ specific tumors in a wide range of experimental animals (Table 1.1).

In contrast to *N*-nitrosamides, which are direct mutagens, *N*-nitrosamines must firstly be metabolised to obtain carcinogenic properties (Figure 1.4). The biochemical activation of *N*-nitrosamines requires the presence of cytochrome P-450. These enzymes can show hydroxylase activity at a carbon adjacent to the nitrosated nitrogen. The intermediate α-hydroxy nitrosamine is chemically unstable and degenerates spontaneously by the elimination of an aldehyde. The remaining alkyldiazohydroxide will produce an agressive alkylating agent, namely a diazonium cation. Luckily, diazonium cations are usually converted to alcohols and alkenes. However a small fraction is able to alkylate DNA, RNA and forms adducts at N-atoms and O-atoms in the bases (Tricker & Preussmann, 1991).

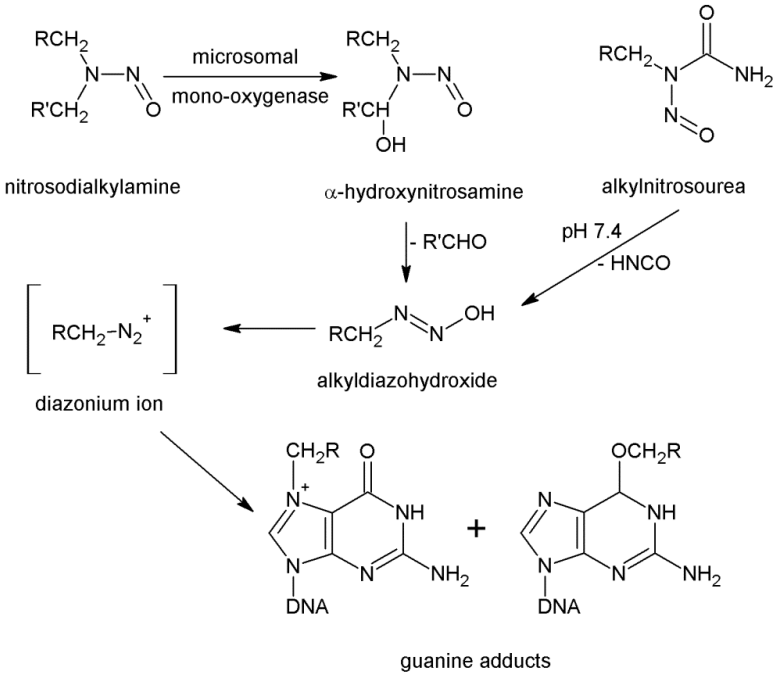


Figure 1.4 Metabolic activation of *N*-nitrosamines (redrafted from Tricker & Preussmann, 1991).

Table 1.1 Overview of some volatile *N*-nitrosamines and their carcinogenicity (redrafted from Tricker & Preussmann, 1991, and IARC, 1978).

N-nitrosamine	Major source	Organotropy[a]	IARC Classification[b]
NDMA	Cured or fried meats, dried fish, shrimps and broiled squid, grain products, dairy and cheese products, dried milk products, edible oils and fats, pickled/fermented vegetables, alcoholic beverages	Liver, kidney, lung	2A
NDEA	Cured meats packed in rubber nettings, grain products, dried cuttlefish	Liver, kidney, lung, oesophagus, (fore)stomach, trachea, nasal cavity	2A
NDBA	Cured meats packed in rubber nettings, smoked chicken, dried fish	Liver, urinary bladder, oesophagus, pharynx, (fore)stomach, respiratory tract, lung	2B
NPYR	Cured meats, fried bacon, pickled vegetables, mixed spices, dried chillies, broiled squid	Liver, nasal cavity, lung	2B
NPIP	Cured meats, fried bacon, peppered salami, pepper and mixed spices, pickled vegetables	Liver, oesophagus, upper respiratory and digestive tracts, nasal cavity (fore)stomach, lung	2B
NMOR	Packaging contamination of fats/margarine and dairy products	Liver, lung, upper respiratory tract, colon	2B

[a]By oral administration to rat, mice and other mammals, [b]IARC classification groups: (1) carcinogenic to humans, (2A) probably carcinogenic to humans, (2B) possibly carcinogenic to humans, (3) not classifiable as to its carcinogenicity to humans, (4) probably not carcinogenic to humans

In DNA, the N^7 and O^6 of guanine (G) and the N^3 and N^7 of adenine (A) are highly susceptible for alkylation. Mainly, the non-mutagenic N^7-guanine adducts are formed, while to a lesser extent the mutagenic O^6-alkylguanine is formed. In normal cells, this lesion is repaired by enzyme O^6-methylguanine-DNA methyltransferase (MGMT). However, MGMT activity can differ among several types of tissues and epigenetic silencing of this enzyme may result in G to A transition mutations in the tumor suppressor gene p53. This may lead to uncontrolled cell division and inhibition of apoptosis and thus induce tumour growth (Esteller et al., 2001).

For ethical reasons, it is impossible to demonstrate the carcinogenic properties of *N*-nitrosamines by the direct administration of these compounds to human volunteers. Therefore, evidence is mainly provided by collecting data from cohort and case-control studies. Meta analyses found relevant associations between high consumption of processed meat and the risk of several cancers, e.g., stomach cancer (Larsson *et al.*, 2006), gastric and esophageal cancer (Jakszyn & Gonzalez, 2006), colorectal cancer (Sandhu *et al.*, 2001; Norat *et al.*, 2002; Larsson & Wolk, 2006; Corpet, 2011), and even childhood brain tumors (Dietrich *et al.*, 2005). In addition, etiological studies demonstrate associations between the occurrence of cancer and the presence of individual *N*-nitrosamines in the diet. For instance, Knekt *et al.* (1999) found an increased risk of colorectal cancer when the estimated intake of NDMA was high. The influence of NDMA on other cancer types is less clear, although Larsson *et al.* (2006) and Mitacek (1999) suggested that the dietary intake of NDMA might also contribute to an increased risk of stomach and liver cancer, respectively. The role of other *N*-nitrosamines on the risk of cancer in humans was more difficult to establish, although some indirect evidence was collected by studying the metabolism of *N*-nitrosamines in cultured human tissues. For instance, human esophageal cancer could be induced by *N*-methyl-*N*-benzylnitrosamine (Shih-Hsin *et al.*, 1986), while NDMA, NMOR, NPIP, and NPYR could provoke hepatoma (Martelli *et al.*, 1988). Based on similarities of the *N*-nitrosamine metabolism in human and animal tissues, the International Agency for Research on Cancer (IARC) made efforts to classify the most common *N*-nitrosamines with respect of the cancer risk for humans (IARC, 1978). The classifications of some common volatile *N*-nitrosamines are included in the last column of Table 1.1.

1.3 SYNTHESIS AND OCCURRENCE OF *N*-NITROSAMINES

1.3.1 GENERAL REACTION MECHANISM

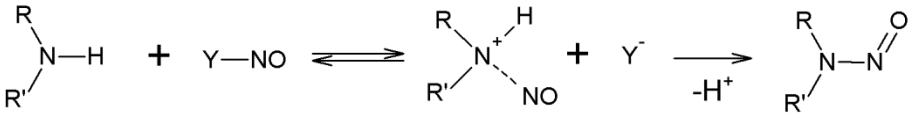


Figure 1.5 *N*-nitrosamine formation from secondary amines (redrafted from Fridman *et al.*, 1971).

N-Nitrosamines are formed by the electrophilic substitution of the amine nitrogen with a nitrosonium cation (NO^+), derived from a nitrosating agent (Y-NO) (Fridman et al., 1971) (Figure 1.5). The *N*-nitrosamines formed from primary amines are not stable and quickly decompose. The intermediate diazonium cation will release nitrogen gas to form alcohols or alkenes (Rostkowksa et al., 1998). Secondary amines and tertiary amines will result in stable *N*-nitrosamines. The nitrosation of tertiary amines is difficult and slow, and proceeds via the intermediate reaction of splitting the molecule in an aldehyde and nitrosable secondary amine (Smith & Pars, 1959).

Many nitrosating agents can be effective nitrosyl donors. In the nitrosation reaction, these nitrosating agents will be transformed to Y-NO form and can be considered to be NO^+ carriers (Bartsch *et al.*, 1988; Loeppky, 1994). The most common sources in the human environment are listed in Table 1.2.

Table 1.2 Some important nitrosating agents that are formed from nitrite and nitrogen oxides (redrafted from Bartsch *et al.*, 1988).

Nitrosating agents	Y-NO	Reaction	Conditions of formation
N_2O_3	O_2N-NO	$2\ HNO_2 \leftrightarrow N_2O_3 + H_2O$	Aqueous, mildly acidic
		$NO + NO_2 \leftrightarrow N_2O_3$	Gas phase, aqueous neutral/alkaline, lipid medium
NO^+	NO^+	$HNO_2 + H^+ \leftrightarrow NO^+ + H_2O$	Aqueous, strongly acidic
$(H_2O)NO^+$	$(H_2O)NO^+$	$HNO_2 + H^+ \leftrightarrow (H_2O)NO^+$	
NOX[a]	X-NO	$H^+ + HNO_2 + X \leftrightarrow NOX + H_2O$	Aqueous, mildly acidic
N_2O_4	O_2NO-NO	$2\ NO_2 \leftrightarrow N_2O_4$	Gas phase
		$2\ NO + O_2 \leftrightarrow N_2O_4$	Lipid medium, aqueous neutral/ alkaline

[a] with X = Cl^-, Br^-, I^-, SCN^-

In foodstuff, and especially in meat products, the main sources of nitrosyl donors are sodium nitrite (by curing) and gaseous nitrogen oxides (by smoking). However, a transformation is necessary to obtain the active nitrosating agent.

In the case nitrite is used at low pH, a proportional amount of its conjugate acid is formed (eq. 1). On its turn, nitrous acid is in equilibrium with dinitrogen trioxide (eq. 2), which provides the nitrosonium cation (NO^+) for the nitrosation reaction of amines (eq. 3) (Mirvish, 1975).

$$NO_2^- + H^+ \overset{\text{pKa 3.36}}{\longleftrightarrow} HNO_2 \quad (1)$$

$$2\ HNO_2 \longleftrightarrow N_2O_3 + H_2O \quad (2)$$

$$R_1R_2NH + O_2N\text{-}NO \longleftrightarrow R_1R_2NNO + HNO_2 \quad (3)$$

To promote the nitrosation reaction, a shift in the equilibrium from the nitrite ion to nitrous acid is necessary by lowering the pH below its pKa of 3.36. However, lowering the pH has an opposite effect on the amount of nonionized amines since an increased protonation occurs. Thereby the basicity of the particular amine plays an important role. The nitrosation of strong basic amines such as piperidine (PIP, pKa = 11.2) and pyrrolidine (pKa = 11.3) is more difficult than this of weak basic amines such as morpholine (pKa = 8.7). Because of the opposite effect of pH on the concentration of unprotonated amine and the concentration of nitrous acid, the optimum pH for the nitrosation is situated between 2.5 and 3.4, dependent of the amine (Bartsch *et al.*, 1988).

Under neutral and alkaline conditions, a fast *N*-nitrosamine formation was observed when basic amines were introduced in an aqueous solution (i.e., at pH 6 – 10, 2 mM amine reacts with a 5-20 fold excess of nitrogen oxide within a few seconds, yielding ca. 10-50% *N*-nitrosamine). In the presence of oxygen, nitric oxide radicals may originate from gaseous nitrogen oxides (e.g., N_2O_3 and N_2O_4). Under anaerobic conditions, powerful nitrosating agents (NOX) are formed from NO in the presence of certain anions (X) (Table 1.2) (Challis & Kyrtopoulos, 1977; Challis *et al.*, 1978).

Furthermore, the nitrosation reaction occurs over a broad temperature range. As expected, the interaction between nitrite and secondary amines increases at high temperatures (above 100 °C). However, *N*-nitrosamine formation still occurs at physiological (24 - 50 °C) and freezing temperatures. During the ice crystallization, the nitrosation reaction is mainly stimulated by concentrated precursors in the remaining liquid phase (Ender & Čeh, 1971).

On the one hand, the formation of *N*-nitrosamines can occur endogenously by the acid-catalyzed reaction in the stomach (Bartsch *et al.*, 1988; Krul *et al.*, 2004). Hereby, thiocyanate, naturally present in saliva, accelerates the formation (Boyland *et al.*, 1971). On the other hand, the consumption of protein-rich foodstuffs remains an important source of exogenously formed *N*-nitrosamines. Therefore human exposure to *N*-nitrosamine contaminated food cannot be neglected.

1.3.2 PRESENCE OF *N*-NITROSAMINES IN FOOD

The awareness of food products being possible exogenous sources of *N*-nitrosamines originates from the early 1960s. In Norway, mink and sheep showed hepatotoxicosis after feeding with nitrite-treated fish meal. The enzymatic breakdown of trimethylamine *N*-oxide (TMAO), the osmoregulator in marine fish, resulted in the formation of dimethylamine and trimethylamine (Fiddler *et al.*, 1991). Unexpectedly, the nitrosation of these amines took place in neutral/alkaline milieu instead of the known acid-catalyzed reaction in the stomach (Lijinsky, 1999) (Figure 1.6).

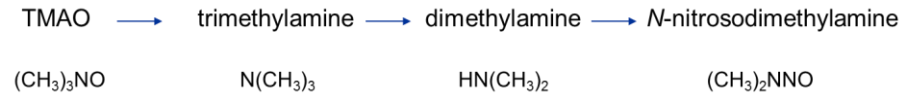


Figure 1.6 Formation of NDMA from the breakdown of TMAO in fish.

In addition, various studies have confirmed the occurrence of several *N*-nitrosamines in exogenous sources, such as tobacco, cosmetics, rubber products and foodstuff. Since 1960s, numerous scientific studies are published wherein the *N*-nitrosamine concentration in various food products was determined. With the purpose to estimate the daily intake of carcinogenic compounds, several food databases were composed. In the framework of the European Prospective Investigation into Cancer and Nutrition (EPIC)-program, Jakszyn *et al.* (2004) compiled a standardized database, based on literature of the period 1980 - 2003, wherein three groups of potential dietary carcinogens, namely *N*-nitrosamines, heterocyclic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs), are listed.

However, Stuff *et al.* (2009), constructed an extensive database which contained more *N*-nitroso compounds and food products. Based on reviewing the literature, 36 *N*-nitroso compounds, including nitrite and nitrate, were recognized. However, 13 compounds were not reported in food products and were related to one particular source. For instance *N*-nitrosonornicotine (NNN), *N*-nitrosoanabasine (NAB) and *N*-nitrosoanatabine (NAT) are tobacco-specific *N*-nitrosamines (Zhou *et al.*, 2007). As can be seen in Table 1.3, only 9 *N*-nitroso compounds of the remaining 23 are frequently detected in food products. In Table 1.3, the individual *N*-nitrosamine concentrations were mostly below 10 µg/kg. However, in some cases, higher levels can be detected. For instance, NPRO and NTCA concentrations can be extremely high. Luckily, these non-volatile *N*-nitrosamines are non-carcinogenic compounds (Tricker & Preussmann, 1991). Although the concentration of NDMA can vary greatly, this simple *N*-nitrosamine is widespread among the different food items. In conclusion, these kind of databases demonstrate that meat products, such as bacon, luncheon meats, sausage, and hot dogs are the richest food sources of *N*-nitroso compounds.

Table 1.3 Overview of the concentrations (µg/kg) of the 9 most occurring *N*-nitroso compounds in a selection of food products (redrafted from (Stuff *et al.*, 2009).

	NDMA	NPYR	NPIP	NDEA	NDBA	NMOR	NPRO	NTCA	NTHZ
Peas		0.25					3.00		
Whole milk	0.14	0.03	0.03						
Margarine	0.26					0.49			
French fries, fried potatoes	0.24	0.41							
White wine	0.25	1.09							
Butter	0.26					0.49			
Refried beans or pinto beans	0.33								
Rolls, buns, muffins, bagels	0.50	0.09		0.23					
Cottage cheese	0.76	0.04	0.09	0.57				15.00	
Fried fish	1.69	0.03		0.88	0.04		12.00	46.60	0.18
Beer	2.02								
Hot dogs or franks	2.21							89.50	3.72
Bacon	4.54	21.29	0.49	0.67	13.65		10.00	1427.42	26.43
Ham	4.90	5.34	0.04	1.49	4.58	2.86	57.52	461.30	1.54
Sauerkraut	6.60	5.55	2.20	0.79			20.95	9.00	
Oysters	11.39	0.38		1.09		0.07	54.16	8.64	
Sausage or chorizo	109.41	0.86	0.01	0.40		0.52	17.20	118.96	1.72

1.3.3 VOLATILE *N*-NITROSAMINES IN MEAT PRODUCTS

1.3.3.1 HISTORY AND OCCURRENCE

In the meat industry, serious concerns emerged when cured bacon samples showed persistent contamination of NPYR, and in lesser extent NDMA, after cooking and frying (Pensabene *et al.*, 1974; Scanlan, 1975). To deal with the *N*-nitrosamine problem, the U.S. Department of Agriculture (USDA), Food and Drug Administration (FDA) and the meat industry started in the late 1960s and early 1970s a cooperative research program. In the same time span, the Canadian Department of Agriculture demonstrated the occurrence of *N*-nitrosamine in premixes containing spices and nitrite curing salt (Sen *et al.*, 1973). In addition, the influence of added sodium nitrite on the formation of NDMA and NPYR in bacon was demonstrated (Sen *et al.*, 1974a). As a result, the use of spice premixes was banned, nitrate (source of nitrite) was eliminated when technologically possible and the addition of nitrite in most meat products was limited to 156 mg/kg. In general, these preventive actions decreased effectively the *N*-nitrosamine levels in meat products. Nevertheless, total elimination of *N*-nitrosamines could not been acquired since the addition of nitrite was inevitable in the course of colour formation and prevention of botulism (Fiddler, 1971). Moreover, cured bacon remained a major issue. The following years, further efforts were made to study *N*-nitrosamine inhibition in meat products by several additives, such as α-tocopherol, ascorbic acid, erythorbic acid and derivatives (Walters *et al.*, 1976; Fiddler *et al.*, 1978; Bharucha *et al.*, 1980). As a result, a specific regulation for cured bacon was composed in the USA, which limited the added amount of nitrite to 125 mg/kg and imposed the mandatory addition of 550 mg/kg sodium ascorbate or erythorbate (McCutcheon, 1984). As a consequence, in one decade, the NPYR levels in bacon decreased from an average value of 63 µg/kg (Fazio *et al.*, 1973) to 21 µg/kg (Sen & Seaman, 1982).

Due to the intensive research in the period 1960s - 1980s, severe *N*-nitrosamine formation in meat products could be mostly avoided, mainly by restricting the added amount of nitrite. Nevertheless, *N*-nitrosamine contamination still occurs. Around the millennium change, there was still concern about the exogenous exposure to *N*-nitrosamine and several surveys have been carried out to obtain insight in the current *N*-nitrosamine contamination in food. In particular, meat products were investigated. In Europe, market surveys were performed in following countries: France (Mavelle *et al.*, 1991), Italy (Gavinelli *et al.*, 1988), The Netherlands (Ellen *et al.*, 1986), Turkey (Ozel *et al.*, 2010), Estonia (Yurchenko & Mölder, 2007) and Poland (Domanska & Kowalski, 2003). Most observations were similar among the different studies and can be mainly summarized based on the results of a report of the European commission (Debeuckelaere, 1999). In this European study, meat products were collected from five European countries: Belgium (99 samples), France (100), Spain (103), Portugal (106) and Italy (99). The most common *N*-nitrosamines, namely NDMA, NDEA, NDBA, NPYR and NPIP were determined. To give a global summary, the

products were divided into five categories according the meat processing (see Table 1.4 and Table 1.5). Whereby typical meat products like dry cured ham and dry fermented sausages are classified in category 2 and 4, respectively.

In Table 1.4, all the categories showed almost identical, high, percentages (72 - 73%) of non-contaminated products. In category five, the proportion of samples showing no contamination (82%) was higher, since nitrite was not added to the fresh meat preparations such as fresh sausages and merguez. However, still 18% contained low concentrations of *N*-nitrosamines. This demonstrated the possible contamination of raw meat materials, which was confirmed by Rywotycki (2003)

Table 1.4 Distribution of the total *N*-nitrosamine contamination rate (%) of meat products categorized according the processing conditions of the meat products (redrafted from Debeuckelaere, 1999).

Category	% total *N*-nitrosamine contamination		
	not detected	≤ 4µg/kg	> 4 µg/kg
1: not comminuted, but heated	72	27	1
2: not comminuted and not heated	73	23	4
3: comminuted and heated	73	26	1
4: comminuted, but not heated	72	23	5
5: fresh meat preparations	82	18	0

In all categories, at least 95% of the samples showed a total *N*-nitrosamine contamination below 4 µg/kg. The highest contamination rate was observed in categories 2 and 4, namely the dried and fermented products.

In Table 1.5 the minimum-maximum range for each individual *N*-nitrosamine is given per product category. The most common *N*-nitrosamines, among the categories, are NDMA and NDEA. However, only low concentrations were measured. The other *N*-nitrosamines were detected in a limited number of samples. Moreover, NDBA was only detected once, namely in a Spanish Salchichón sample (category 4). Although the number of NPIP and/or NPYR contaminated samples is small, some concern is needed since the concentrations can be high. For instance, in a liver paste sample (category 3) both NPYR and NPIP at concentrations of 10.5 and 43.6 µg/kg, respectively, were measured. In category 3, almost 7% of the samples were contaminated with NPIP, whereof two dry fermented sausages of the Belgian market contained NPIP concentrations above 20 µg/kg.

The European study only included the European market and thus over 80% of the investigated meat products were made of pork meat. Meat products containing other kinds of meat, e.g., beef, poultry, mutton, or mixtures thereof, were scarce. In contrast, the study performed in Turkey (Ozel *et al.*, 2010) investigated the *N*-nitrosamine contents of some traditional (halal) products (Table 1.6). Instead of pork meat, other meat sources were used; e.g., a mixture of beef and sheep fat in sucuk (i.e., a traditional dried sausage) and mainly poultry in salami.

Table 1.5 *N*-Nitrosamine levels (µg/kg, min-max) measured in the different categories of meat products (redrafted from Debeuckelaere, 1999).

		N-nitrosamine concentrations (µg/kg)[a]				
Category	n	NDMA	NDEA	NDBA	NPYR	NPIP
1: not comminuted, but heated	79	nd[b] - 2.2	nd - 5.5	nd	nd	nd
2: not comminuted and not heated	177	nd - 5.9	nd - 3.7	nd	nd - 3.0	nd - 13.0
3: comminuted and heated	85	nd - 1.8	nd - 3.7	nd	nd - 10.5	nd - 43.6
4: comminuted, but not heated	148	nd - 6.3	nd - 6.3	nd - 2.8	nd - 2.0	nd - 29.0
5: fresh meat preparations	18	nd	nd - 0.6	nd	nd	nd - 2.0

[a]min - max range, [b]nd: not detected

In general, the results are similar to these of the European study. The most interestingly results were obtained from the analyses of the doner kebab samples. Although this type of product is made without nitrite, the samples showed, after the conventional roasting with a gas flame grill, high *N*-nitrosamine contamination. Certainly the NPYR and NPIP concentrations were surprisingly high.

Table 1.6 Concentration ranges of some volatile *N*-nitrosamines found in common Turkish meat products (redrafted from Ozel *et al.*, 2010).

Product	*N*-nitrosamine concentrations (µg/kg)[a]				
	NDMA	NDEA	NDBA	NPYR	NPIP
Sucuk	nd[b]-0.8	nd-1.0	nd-1.7	0.1-1.4	0.2-2.7
Sausage	nd-1.2	nd-1.7	nd	nd-0.3	nd-0.7
Salami	nd-0.3	nd-0.3	nd-0.6	nd-0.5	0.2-1.4
Doner Kebab	nd-1.1	0.1-1.0	nd	0.4-7.7	0.2-7.2

[a]min - max range (n = 6), [b]nd: not detected

Also in other continents, some traditional meat products can be found. For example, the Chinese kitchen is famous for its “Peking” duck, i.e., duck coated with sugar and flavourings and afterwards roasted. In the study of Song and Hu (1988), the roasted ducks showed comparable concentrations of NDMA and NDEA as the sausage samples (Table 1.7). Although the opposite was expected due the roasting of the duck, no NPYR formation was observed. In the sausage samples, high NPYR concentrations were measured. Since Chinese sausages (Lap cheong) is a general name for sausages produced in China, it can contain pork meat as well as liver (from pork, duck or turkey) and blood. Unfortunately, the ingredients and processing conditions were not described in the study and so it is difficult to explain the strong NPYR contamination.

Table 1.7 Concentration ranges of NDMA, NDEA and NPYR found in a selection of meat products from the Chinese market (redrafted from Song & Hu, 1988).

Product	n	*N*-nitrosamine concentrations (µg/kg)[a]		
		NDMA	NDEA	NPYR
Sausage	194	nd - 7.4	nd - 6.7	nd - 14.0
Ham	15	nd - 4.0	nd - 1.2	nd
Roast duck	4	nd - 7.4	nd - 3.0	nd
Luncheon meat	3	nd	nd	nd
Others	55	nd - 1.2	nd	nd

[a]min - max range

As discussed in this chapter, *N*-nitrosamines can occur in varying concentrations in several types of meat products. In many cases, the occurrence of each individual *N*-nitrosamine in the product can be related to specific process conditions and ingredients.

1.3.3.2 FORMATION IN MEAT PRODUCTS

***N*-Nitrosopyrrolidine**

Despite the nitrite limiting actions, bacon could still, though sporadically, contain high NPYR concentrations (up to 65 µg/kg) (Canas *et al.*, 1986). Hereby, intensive heating, namely frying, plays a key role in the formation. At elevated temperatures, the collagen of the connective tissue hydrolyzes to produce the free amino acid proline. At temperatures of 100 - 150 °C, NPYR is formed mainly via the not carcinogenic intermediate NPRO. On the other hand, at temperatures higher than 175 °C, proline is more likely to decarboxylate to pyrrolidine prior to nitrosation (Nakamura *et al.*, 1976) (Figure 1.7). Drabik-Markiewicz *et al.* (2009, 2010) confirmed the contribution of pyrrolidine and proline to the NPYR formation during intensive heating of the cured meat. On the contrary, hydroxyproline, an amino acid which is structurally almost similar to proline, did not result in an increased concentration of NPYR. Neither during the heating of a liquid oil-water model (simulating the frying of bacon) (Bills *et al.*, 1973) as during the heating of a lean meat model (Drabik-Markiewicz *et al.*, 2009, 2010) NPYR formation could be observed.

Biogenic amines, derived from the decarboxylation of amino acids, are also considered to contribute to the formation of *N*-nitrosamines. As mentioned in section 1.3.1, the primary amines, PUT and SPD, are normally not expected to form *N*-nitrosamines. The contact with nitrite leads to the diazotization and deamination of the amines, resulting in the formation of alcohols and alkenes (Rostkowksa *et al.*, 1998). Nevertheless, in vitro studies of oil-water models or low-moisture systems, containing nitrite and amines (ratio 2:1) (Bills *et al.*, 1973; Warthesen *et al.*, 1975) demonstrated the formation of small amounts of NPYR from PUT and SPD during intensive heating (160 - 170 °C). In Figure 1.8, the possible reaction mechanism of PUT is given. It is suggested that at high temperature, primary amines undergo a deamination and cyclization reaction forming secondary amines, prior to nitrosation (Lijinsky &

Epstein, 1970). In this way, primary amines may still indirectly be precursors of *N*-nitrosamines.

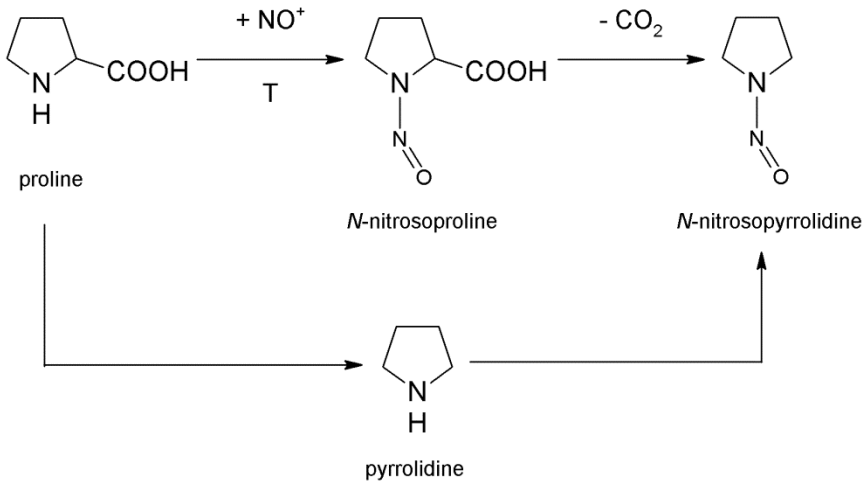


Figure 1.7 Formation of NPYR from the amino acid proline in fried bacon (redrafted from Kikugawa & Kato, 1991).

However, it can be questioned if model systems adequately reflect realistic conditions during food processing. Hwang and Rosen (1976) could not reproduce these observations during the pan-frying of bacon. Drabik-Markiewicz (2011) demonstrated that heat treatments, ranging from 85 °C to 220 °C, of lean meat, could not provoke the formation of NPYR from PUT or SPD. In addition, Hildrum (1975) suggested the possible formation of dozens of different *N*-nitroso compounds from SPD and SPM, since both molecules possess secondary amine groups which are all conceivable for nitrosation. In this study, ɣ-butenyl(3-propenyl)nitrosamine (BPN) was identified as the principal *N*-nitrosamine from SPD, and in much lesser extent NPYR was detected (Figure 1.9).

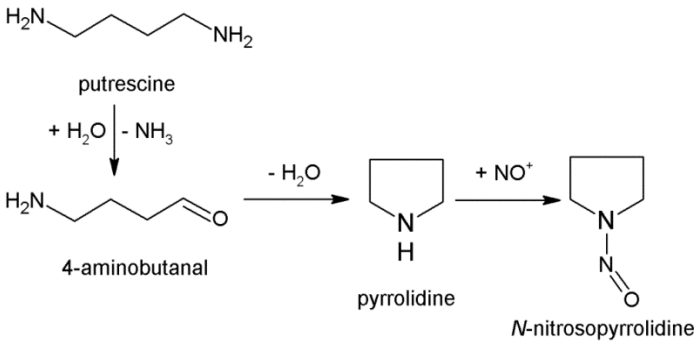


Figure 1.8 Possible formation of NPYR from the biogenic amine PUT.

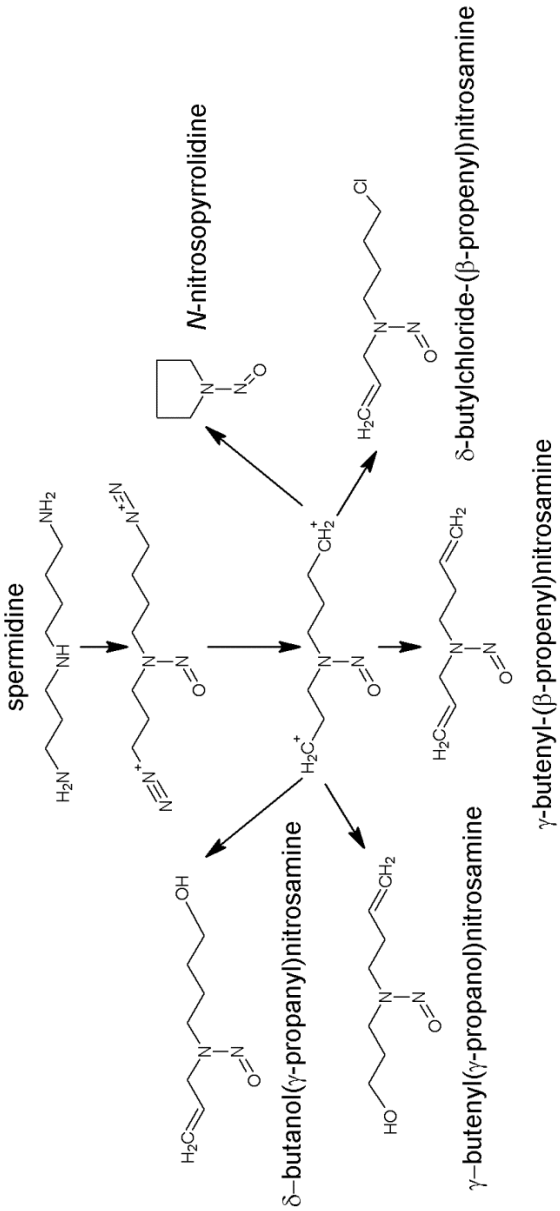


Figure 1.9 Pathway of the formation of nitrosamines from the nitrosation of spermidine (redrafted from Hildrum, 1975).

N-Nitrosopiperidine

Another *N*-nitrosamine often linked to the presence of biogenic amines is NPIP. As schematically shown in Figure 1.10, Warthesen (1975) demonstrated in a high temperature-low-moisture model that a mixture of nitrite and CAD yields considerable amounts of NPIP. Moreover, the parental amino acid, lysine, can act as precursor of NPIP. However, it must be emphasized that in this study mainly *N*-nitrosopipecolic acid (NPCA) was formed from lysine.

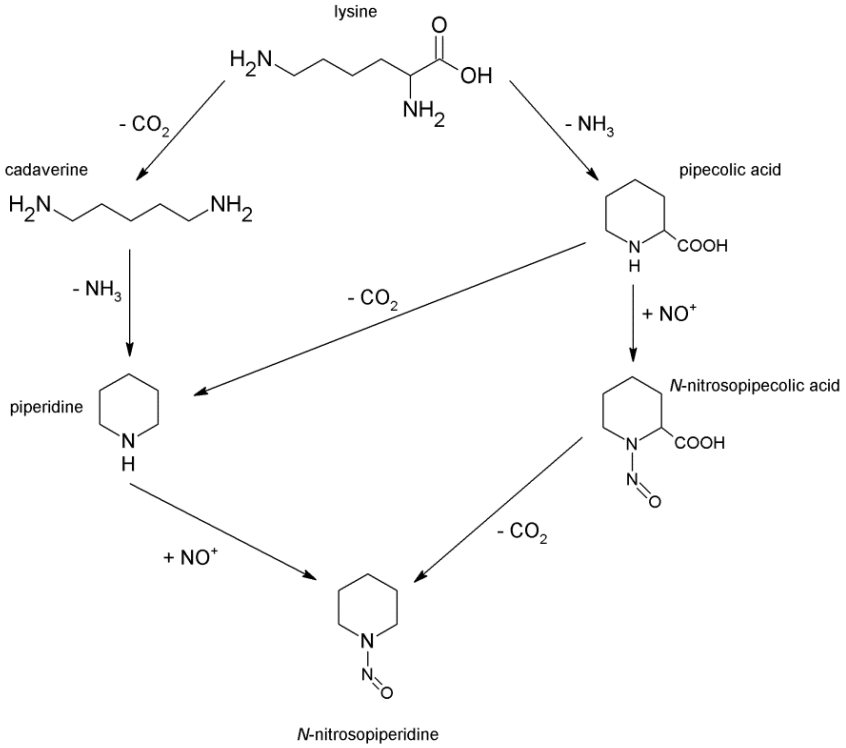


Figure 1.10 Possible routes for the formation of NPIP from lysine and CAD (redrafted from Warthesen *et al.*, 1975).

Reports in which the formation of NPIP from CAD was studied in meat products are scarce. However, some critical approach is appropriate since, as mentioned already for NPYR, conflicting results were obtained in the studies in liquid models versus realistic meat models. As far as we know, only Drabik-Markiewicz *et al.* (2011) investigated the contribution of CAD to the formation of NPIP in meat systems. In contrast to NPYR, it was confirmed in this study that CAD can indeed be converted to NPIP during intensive heating (above 160 °C) of the lean meat model.

Alternatively, the formation of NPIP can be related to the introduction of the direct precursor PIP to the meat product. In this case, spices could be the main source of PIP and piperidine-derivatives, such as piperine (Nakamura *et al.*, 1981). In fact, many studies suggest the relation between a high NPIP contamination rate and strong seasoning of the meat products (Yamamoto *et al.*, 1984; Domanska & Kowalski, 2003; Yurchenko & Mölder, 2007)

***N*-Nitrosodimethylamine**

NDMA is the most commonly occurring *N*-nitrosamine in food products in general. However, the source of the precursors dimethylamine and trimethylamine can differ greatly among the different food categories. For instance, the NDMA pathway in beer originates from the reaction of tertiairy alkaloids, such as gramine and hordenine, with nitrogen oxides of the combustion smoke during the direct-fire drying step of the malting process (Mangino & Scanlan, 1985). In contrast, the sources of NDMA in meat products are more difficult to determine. Possibly, the major source of trimethylamine and dimethylamine in meat is choline, a compound of the lecithin fraction in animal tissue. The increased NDMA contamination in heated meat products is mainly caused by the thermal degradation of lecithin to choline and subsequently to trimethylamine. After demethylation, dimethylamine can be nitrosated to yield NDMA (Figure 1.11) (Pensabene *et al.*, 1975).

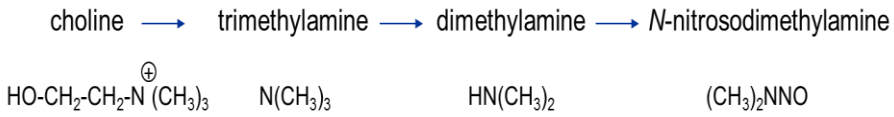


Figure 1.11 Formation of NDMA from choline during the heating of meat products (redrafted from Pensabene *et al*, 1975).

On the one hand, high concentrations of choline can be present by the use of organ meats in comminuted products. As a result, edible offals processed meat products can be highly contaminated with NDMA. For instance up to 30.1 µg/kg NDMA can be measured in blood sausages and liver sausages (Domanska & Kowalski, 2003). On the other hand, lecithin, mainly derived from soybean or egg yolk, can be added to meat products as emulsifying agent. The NDMA pathway was confirmed by heating model systems containing nitrite and lecithin (Pensabene *et al.*, 1975). However, it is uncertain whether only lecithin contributes to the NDMA formation in meat products since the dimethyl-unit is a part of many molecules present in animal tissue (Spinelli-Gugger *et al.*, 1981). For instance, up to 63 µg/kg NDMA can be measured in aspic (gelatin containing) meat product such as souses (head cheeses pickled in vinegar) (Fiddler *et al.*, 1975). In addition, Drabik-Markiewicz (2011) demonstrated the contribution of the biogenic amines spermidine (SPD) to the NDMA formation at processing temperatures above 160 °C.

N-Nitrosothiazolidine and *N*-nitrosothiazolidine-4-carboxylic acid

Various smoked meats including bacon, dry sausages and pork bellies can contain NTHZ and the carboxylated variant NTCA (Mandagere *et al.*, 1984; Pensabene & Fiddler, 1985). Sen *et al.* (1986) measured extremely high concentrations of NTCA (up to 14 000 µg/kg) in bacon. Luckily, it seems that NTHZ and NTCA are non-carcinogenic compounds (Lin & Gruenwedel, 1990; Tricker & Preussmann, 1991). The formation is related to the smoking of meat products. Smoke, either wood smoke or liquid smoke condensates, contains formaldehyde and acetaldehyde which can react with cysteine, cysteamine or cystine of the protein-rich meat product (Ikins, *et al.*, 1988). Subsequently the nitrogen of the thiazolidine ring can be nitrosated by the nitrite added for curing the meat. In Figure 1.12, the formation of NTHZ from the reaction of formaldehyde and cysteamine is represented schematically.

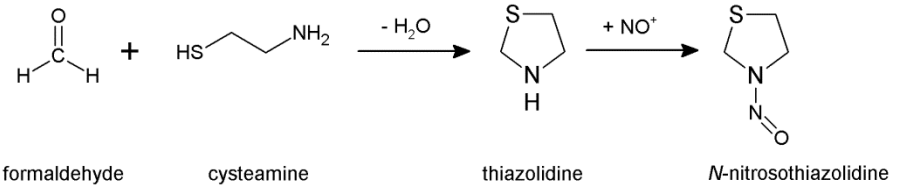


Figure 1.12 Formation of NTHZ from formaldehyde and cysteamine when nitrite cured meat is smoked (redrafted from Ikins *et al.*, 1988).

N-nitrosodiethylamine, *N*-nitrosodibutylamine and *N*-nitrosomorpholine

Most nitrosatable secondary amines are foodborne and can be naturally found in the meat products. Nevertheless, exceptionally, *N*-nitrosamine contamination can occur by the migration of precursors from the packaging material to nitrite cured meat products. In the late 1980s, it was discovered that the occurrence of NDBA, and to lesser extent NDEA, could be traced back to the use of accelerators, e.g., zinc dibutyl- and diethylcarbamate, during the vulcanization process of the rubber materials, such as nettings and casings (Sen *et al.*, 1993). In this way, up to 64 µg/kg NDBA was measured in the outer layer of the meat product, while in the core, only traces (less than 1 µg/kg) were detected. The rubber industry introduced an alternative amine additive as accelerator, namely zinc dibenzyl- instead of zinc dibutylcarbamate. Although the NDBA issue was solved, the use of the reformulated nettings resulted in the formation of *N*-nitrosodibenzylamine (NDBzA). As a consequence, a survey showed that more than 50% of the investigated netted hams contained in the outer layer more than 100 ppb NDBzA. To overcome this problem, diisobutyldithiocarbamate was suggested as alternative accelerator since the nitrosation is reduced due steric hindrance (Fiddler *et al.*, 1998).

Sporadically, meat products can contain traces of NMOR. In heated meat products up to 2 µg/kg and in smoked sausage up to 7 µg/kg NMOR was detected (Mavelle *et al.*, 1991). However, the direct precursor morpholine does not occur naturally in food products. Also here, the contamination of the meat products can be considered to come from external sources such as packaging materials. For instance, Sen and Baddoo (1986) demonstrated that the NMOR content in margarine finds its origin in the waxes of the paper wrappings. Also, rubber packaging materials show high NMOR contents (Kataoka *et al.*, 1997). In addition, anticorrosion agents, used in steam installations, can contain morpholine. Unintended contamination with these anticorrosion agents during the processing can result in the formation of NMOR in meat products (Domanska & Kowalski, 2003).

1.4 RISK OF *N*-NITROSAMINE FORMATION IN DRY FERMENTED SAUSAGES

In the previous section it was clear that meat products, especially the ones which were comminuted, but not heated, can contain a diversity of *N*-nitrosamines in variable amounts. Dry fermented sausages can indeed contain the necessary precursors. In first instance, secondary amines can be abundantly present in the form of biogenic amines, which is the subject of Chapter 1.4.1. Secondly, nitrosating agents can be introduced in the meat products, either from the added nitrite/nitrate or nitrogen gases produced during the smoking. In the previous section, heating was mentioned several times as an important factor in the formation of *N*-nitrosamines. However, as can be seen in the flow diagram (Figure 1.13), no heating step is included in the production of the dry fermented sausages. Nevertheless, several other factors, e.g., ingredients, additives and production steps (e.g., fermentation), can influence the formation of *N*-nitrosamines as well, as discussed in Chapter 1.4.2.

1.4.1 PRESENCE OF BIOGENIC AMINES

1.4.1.1 SYNTHESIS AND OCCURRENCE

In meat products, the formation of biogenic amines has mainly been related to the activity of micro-organisms. The major formation pathway is the decarboxylation of free amino acids by enzymatic activity of microflora (Suzzi & Gardini, 2003). As a result, the corresponding basic amines of the amino acid can accumulate during fermentation. Microbial biogenic amine production has the aim to generate proton motive force and protect itself against intracellular (and extracellular) acidification (Molenaar *et al.*, 1993). In addition, it has also been suggested as an alternative pathway for energy generation since the biogenic amine production increases under poor growth conditions when the fermentable carbohydrates are exhausted (Konings *et al.*, 1997).

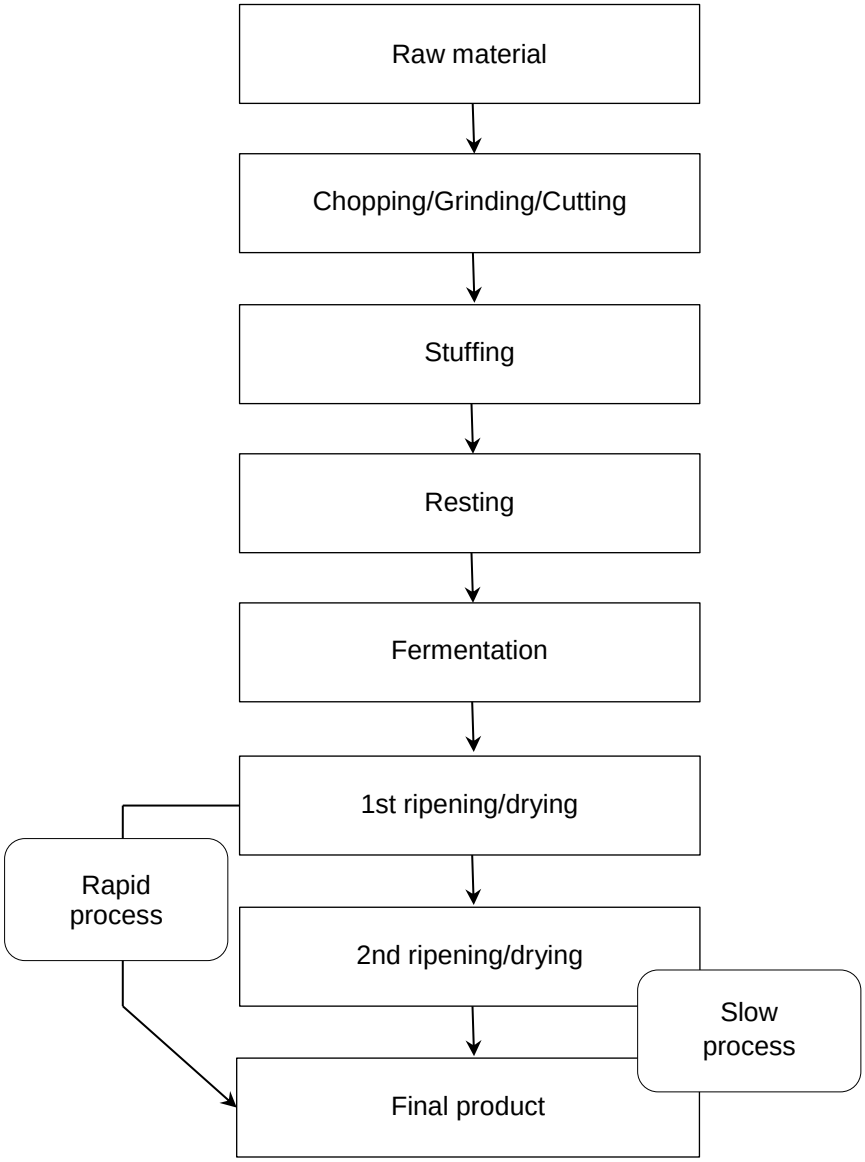


Figure 1.13 Major steps in the flow diagram of dry fermented sausages (redrafted from Toldrà, 2002).

As a consequence, fermented meat products can be contaminated with histamine (HIS), TYR, tryptamine (TRYP), PUT, CAD. In a lesser extent phenylethylamine (PHE), serotonin (SER) and agmatine (AGM) can also be present. Furthermore, meat products contain the physiologically natural polyamines SPD and spermine (SPM), which are not associated with microbial activity. In contrast, their biosynthesis can be described as a more complex process of meat-born endogenous enzymatic reactions (Latorre-Moratella *et al.*, 2008). In

Figure 1.14, an overview of biogenic amines formation from their analogous amino acids in meat is given.

For safety and quality purposes, lots of studies have been carried out to investigate the occurrence and formation of biogenic amines in dry cured meat products. A selection of market surveys of dry fermented sausages is collected in Table 1.8. The most abundant biogenic amine is TYR, generally representing 38 to 62% of the total biogenic amine amount (Demeyer *et al.*, 2000; Latorre-Moratella *et al.*, 2008). Hereby, lactic acid bacteria (LAB), e.g., *Lactobacillus* spp., *Carnobacterium* spp., *Enterococcus* spp., play an important role since they possess strong tyrosine decarboxylase (TDC, EC 4.1.1.25) activity (Moreno-Arribas & Lonvaud-Funel, 1999). In addition, phenylalanine decarboxylase (PDC, EC 4.1.1.53), found in *Staphylococcus* sp., *Micrococcus* sp. and *Sarcina* sp. (Nakazawa *et al.*, 1977), showed also decarboxylase activity towards phenylalanine, histidine, tryptophan and 5-hydroxytryptophan. As a result, PHE, HIS, TRYP and SER, respectively, are sporadically found in dry cured meat (Table 1.8) (Lorenzo *et al.*, 1999; Latorre-Moratella *et al.*, 2008). However, HIS can also be formed by the activity of a specific histidine decarboxylase enzyme (HDC, EC 4.1.1.22). In meat products, mostly Gram-positive bacteria used as starter culture or present as spoilage bacteria, such as *Clostridium perfringens*, show HDC activity (Landete *et al.*, 2007). Although HIS is less frequently detected and at much lower concentrations than TYR, occasionally extreme HIS values (up to 650 mg/kg) have been reported (Coïsson *et al.*, 2004).

Besides the aromatic and heterocyclic amines described above, the aliphatic diamines, CAD and PUT can also be regarded as ‘true’ biogenic amines. Other aliphatic polyamines such as SPD, SPM and AGM are indicated as natural polyamines (Bardocz, 1995). The synthesis of CAD is mainly catalyzed by lysine decarboxylase (LDC, EC 4.1.1.18) which can be found in both Gram-negative bacteria, mainly *Enterobacteriaceae*, and Gram-positive bacteria such as *Clostridium* spp., *Listeria* spp and staphylococci (Landete *et al.*, 2007). PUT is derived by the activity of ornithine decarboxylase (ODC, EC 4.1.1.17), an enzyme being produced by Gram-positive bacteria, e.g., *Lactobacillus* spp., and Gram-negative bacteria, e.g., *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio Cholera*, ,… (Landete *et al.*, 2007). Ornithine is not a proteinic amino acid but results from enzymatic

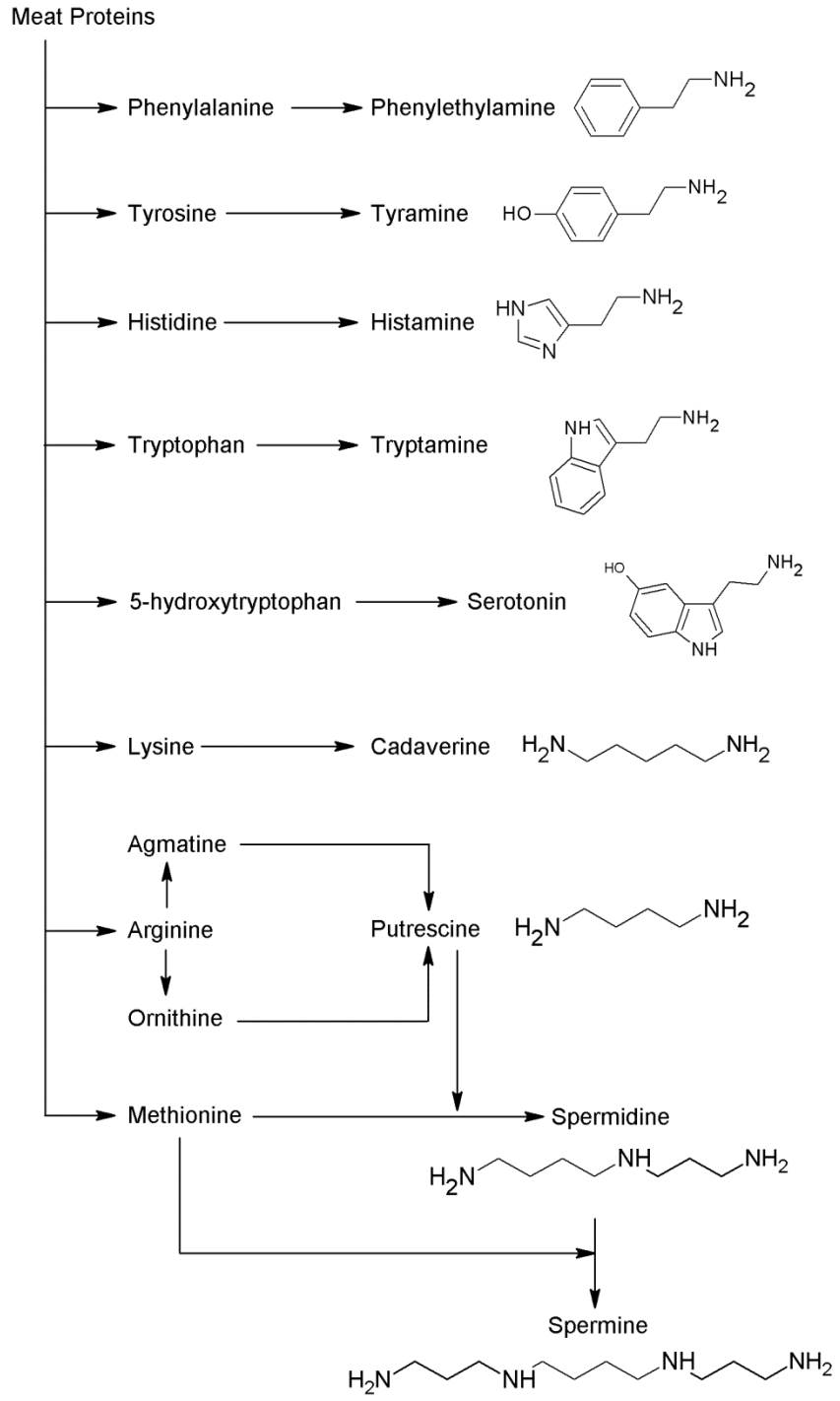


Figure 1.14 Formation of biogenic amines.

hydrolysis of the semi-essential amino acid arginine by meat-born arginase (EC 3.5.3.1) (ten Brink *et al.*, 1990). Alternatively, PUT can be formed microbiologically from arginine by the intermediate AGM (Shaibe *et al.*, 1985). Furthermore, PUT, can play a role as N-acceptor in the formation of SPD and SPM from methionine (Bowman *et al.*, 1973). In dry fermented meat products, PUT and CAD are, after TYR, the most abundant biogenic amines (Table 1.8). SPD and SPM, however, are naturally present in meat at low concentrations. During the fermentation phase, SPD and SPM levels remain constant, while throughout the drying and ripening phase even a slight decrease can occur (Parente *et al.*, 2001). This reduction is most likely due to the deamination by microbial polyamine oxidase (PAO; EC 1.5.3.11) (Razin *et al.*, 1959).

1.4.1.2 TOXICOLOGY

Concerning the toxicology, the consumption of meat products contaminated with biogenic amines is under normal conditions not a health risk. In general, healthy consumers are protected by intestinal and hepatic barriers, mainly mono- and diamine oxidases (MAO; EC 1.4.3.4, and DAO; EC 1.4.3.6, respectively). Low amounts of biogenic amines can be metabolised to physiologically less active degradation products (Ruiz-Capillas & Jimenez-Colmenero, 2005).

Nevertheless, aromatic and heterocyclic amines are all vasoactive amines and represent a food poisoning hazard, especially when the consumption of contaminated food is coupled with the intake of amine oxidase-inhibiting drugs, alcohol, other amines or by susceptible persons suffering from gastrointestinal diseases (Taylor, Other microbiological intoxications, 1990). Vasopressors, like TYR and PHE cause an increased blood pressure and may induce severe headaches, brain haemorrhage and even heart failure (Til *et al.*, 1997). TYR poisoning, better known as the ‘cheese reaction’, was primarily seen in patients combining the consumption of cheese and the intake of mono amine oxidase inhibitors (MAOI) drugs, such as antidepressants (Smith, 1980). Dry fermented sausages can contain similar amounts of TYR as cheese. Even for healthy people, the intake of high loads of biogenic amines may make the detoxification system unable to eliminate all the bioactive amines (Bodmer *et al.*, 1999). Therefore, maximum allowable levels between 100 and 800 mg/kg have been proposed for TYR and 30 mg/kg for PHE (Silla Santos, 1996).

In contrast to the toxicological effect of TYR and PHE, HIS causes a decrease in blood pressure and results in allergy-like symptoms such as headaches, heart palpitations, edema, vomiting, diarrhea,…. The hypersensitivity to HIS could appear when DAO enzyme activity in the intestinal tract, is affected by genetic deficiencies or by the use of DAO inhibiting (DAOI) drugs (Bardocz, 1995). Depending on the intake, HIS may cause slight (8 – 40 mg), intermediate (40 – 100 mg) or intensive (100 mg) food poisoning (Maijala & Eerola, 1993). Worldwide, HIS food poisoning still

Table 1.8 Biogenic amine and polyamine contents (mg/kg) in commercial fermented sausages.

Type	TYR	PHE	HIS	TRYP	PUT	CAD	SPD	SPM
Finnish sausage[a]	88[i] (4-200)[j]	13 (2-248)	54 (nd-180)	14 (nd-43)	79 (nd-230)	50 (nd-270)	4 (2-7)	31 (19-46)
Norwegian sausage[b]	17	1	1	14	nd	1	nd	4
Belgian[b]								
sausage (north)	70	5	2	18	28	nd	4	30
sausage(south)	160	22	36	56	100	6	5	30
German Cervelat[c]	121	-[l]	52	-	242	-	9	38
French Saucisson[d]	164 (84-217)	1 (nd-4)	15 (15-16)	nd	223 (61-317)	71 (39-110)	4 (2-6)	84 (82-86)
Italian								
Salimini[e]	205 (60-372)	14 (nd-53)	46 (8-165)	20 (nd-69)	-	-	-	-
Salsiccia[f]	77 (nd[k]-339)	nd	nd	-	20 (nd-78)	7 (nd-39)	19 (nd-57)	3 (nd-28)
Soppressata[f]	178 (0-557)	3 (nd-20)	22 (nd-101)	-	99 (nd-416)	61 (nd-271)	40 (nd-91)	36 (nd-98)
Spanish[g]								
Chorizo	282 (30-627)	1 (nd-52)	18 (nd-314)	16 (nd-88)	60 (3-416)	20 (nd-658)	4 (2-10)	26 (14-44)
Fuet	191 (32-743)	2 (nd-34)	2 (nd-358)	9 (nd-68)	72 (2-222)	19 (5-51)	5 (1-11)	17 (9-30)
Salchichon	281 (53-513)	7 (nd-35)	7 (nd-151)	9 (nd-65)	103 (6-400)	12 (nd-342)	5 (1-14)	15 (7-43)
Sobrasada	332 (58-501)	2 (nd-39)	9 (3-143)	12 (nd-65)	65 (2-501)	13 (3-42)	3 (2-7)	14 (10-18)
Turkish suçuk[h]	75 (2-676)	3 (nd-20)	19 (nd-136)	7 (nd-82)	56 (nd-364)	27 (nd-199)	3 (nd-11)	9 (nd-16)

[a]Eerola *et al.* (1998), [b]Demeyer *et al.* (2000), [c]Treviño *et al.* (1997), [d]Montel *et al.* (1999), [e]Coïsson *et al.* (2004), [f]Parente and Suzzi (2001), [g]Hernández-Jover *et al.* (1997), [h]Gencçelep *et al.* (2008), [i]mean value, [j](minimum-maximum), [k]nd: not detected, [l]–: not analysed.

occurs and is mainly caused by the consumption of scombroid fish, mainly tuna and mackerel (Lehane & Olley, 2000). Therefore, a maximum level of 100 mg/kg HIS in some fish species is imposed by legal regulations (Commission regulation (EC) No 2073/2005). Although in dry fermented meat products comparable concentrations (Table 1.8) can be detected as found in processed scombroid fish products (15 – 54 mg/kg), no regulation concerning maximum levels in meat products exists (Taylor & Leber, 2007).

The aliphatic polyamines are only toxic in high concentrations, i.e., no-observable-adverse-effect-levels (NOAEL) are 180, 83 and 19 mg/kg of body weight for diamines (CAD and PUT), SPD and SPM, respectively (Larqué *et al.*, 2007). Although the concentration range of the PUT and CAD found in foodstuff is not toxic, those amines aggravate the adverse effects of HIS and TYR as they compete for the detoxification system (Bardocz, 1995). Despite the fact that aliphatic polyamines are not directly toxic, they are regularly investigated as they are considered to be precursors of the carcinogenic *N*-nitrosamines (see Chapter 1.3.3.2).

1.4.1.2 FACTORS INFLUENCING THE BIOGENIC AMINE FORMATION

The variability of biogenic amines in dry fermented meat products is large. Differences in biogenic amine accumulation is the result of a combination of multiple factors during the dry cured meat processing, e.g., the quality of the raw material, the presence and growth of decarboxylase positive bacteria, variations in sausage formulations and processing conditions.

Raw meat materials

The intrinsic quality of the used raw materials is important for the production of dry fermented sausages. Sausages prepared from poor quality raw materials can lead to high amounts of biogenic amines in the end product (Latorre-Moratella *et al.*, 2008). The accumulation of PUT, CAD, HIS and TYR is mostly related to the presence and growth of *Enterobacteriaceae* and *Pseudomonaceae* in the raw meat (Bover-Cid *et al.*, 2000, 2003). Although these bacteria are out-competed in the first stage of the fermentation, the bacterial enzymes can still be present and decarboxylate the free amino acids formed during fermentation and ripening (Shalaby, 1996; Roig-sagués *et al.*, 1996). Besides the overall suggestion to start with raw meat materials under strictly hygienic conditions, some authors have tried to reduce the initial contaminating flora. Obviously, the reduction of these bacteria cannot be established by pasteurisation nor sterilization because the raw material will change irreversible, both sensorially and technologically. However, other techniques, such as high pressure (Latorre-Moratalla *et al.*, 2007) and freezing, can be used to reduce the loads of potential aminogenic bacteria prior to fermentation. Under correct freezing conditions microbial reduction, particularly of Gram-negative bacteria, takes place and results in a decrease of CAD. However, PUT, also known to be produced by *Enterobacteriaceae*, is not influenced. Moreover, the TYR production increases when frozen meat materials are used, since freezing contributes to the selection of Gram-positive LAB and catalase positive cocci (Bover-Cid *et al.*, 2006).

Starter culture

Although it is widely assumed that LAB are the main producers of biogenic amines (Silla Santos, 1996), the accumulation can be prevented by selecting a decarboxylase free starter culture (Maijala & Eerola, 1993). Strains of *L. sakei, L. bavaricus* and *L. plantarum* have lower aminogenic activity (Aymerich *et al.*, 2006; Ammor & Mayo, 2007). Moreover, the addition of a starter culture can prevent the uncontrolled growth of decarboxylase positive contaminants (Suzzi & Gardini, 2003). But the effectiveness of the starter is strongly conditioned by the quality of the raw materials and the technological conditions, necessary for a fast development of the starter. As a result, a number of studies failed to demonstrate the efficiency of selected LAB in real fermentation processes (Buncić *et al.*, 1993; Maijala *et al.*, 1995a; Bover-Cid *et al.*, 1999a; Ayhan *et al.*, 1999; Bozkurt & Erkmen, 2002).

Besides LAB, used for the acidification, most starter mixes contain also micrococci which can reduce nitrate into nitrite and are important for the colour formation. In general staphylococci, micrococci and kocuriae are described as weak decarboxylase active microorganisms (Montel, 1999; Martuscelli *et al.*, 2000; Bover-Cid *et al.*, 2001b; Martín *et al.*, 2006). However, some species of the genera *Micrococcus* and *Staphylococcus* produce HIS (Silla Santos, 1996; Suzzi & Gardini, 2003). Most *S. xylosus* strains are not able to decarboxylate ornithine and lysine, while *S. carnosus*, isolated from starters show ornithine decarboxylase activity (Komprda *et al.*, 2004).

Ingredients and additives

The presence of salt in the meat product has direct and indirect effects on the ability of decarboxylation. Firstly, salt acts as a hurdle, inhibiting the growth of decarboxylase positive contaminating flora, which is generally not halo-tolerant. Secondly, the activity of these enzymes is dependent of the salt content and can be disturbed starting from levels of 3.5–5.5% NaCl (Suzzi & Gardini, 2003).

Nitrite and sulphite are generally known to inhibit the growth of food pathogens, and also the inhibition of *Enterobacteriaceae* in dry fermented sausages was proven (Bover-Cid *et al.*, 2001a; González & Díez, 2002; Gençcelep, 2007). This may result in a drastic reduction of the biogenic amine content, mainly PUT and CAD. Although these additives inhibit the growth of *Enterobacteriaceae,* the influence on other bacteria can be diverse. The growth of some bacterial populations may be promoted by the absence of the *Enterobacteriaceae*. For instance, the addition of 150 mg/kg sodium nitrite reduced the amount of PUT and CAD, but the amount of HIS increased substantially (Bozkurt & Erkmen, 2007; Gençcelep, 2007). The same effect can be achieved by the use of 500 mg/kg nitrate in combination with a nitrate reducing starter (Ayhan *et al.*, 1999). In contrast, the addition sodium sulphite in concentrations above 500 mg/kg inhibited the CAD accumulation, but PUT and TYR levels increased (Bover-Cid *et al.*, 2001c;

Komprda *et al.*, 2004). Nevertheless, the use of sulphite is not allowed in dry fermented sausages (Directive 2006/52/EC, 2006).

Besides the use of antimicrobial additives, glucose can be used in the meat batter as a primary growth substrate for the starter culture. In this way the lag-phase is shortened, whereby the LAB can dominate and out-compete contaminating microflora (Toldrá, 2007). Sugar omission results in a higher TYR and CAD formation due to the slower growth of LAB and thus the delayed reduction of *Enterococcaceae* and *Enterobacteriaceae* (Bover-Cid *et al.*, 2001a).

Processing
During the fermentation, the product must undergo a rapid acidification to inhibit the growth of contaminating, possible aminogenic, bacteria (Maijala *et al.*, 1995b; Bover-Cid *et al.*, 2001a). On the condition that raw meat of good hygienic standards is used, *Enterobacteriaceae* will not develop and CAD will not be formed. Paradoxically, fast acidification must be stimulated but the final pH may not be too low, because biogenic amine formation is a protective action against acidic environment (Molenaar *et al.*, 1993). However, most fermented meat products have a pH-value closely to the pH-optimum of amino decarboxylases, i.e., between 4.0 and 5.5 (Silla Santos, 1996).

During the ripening, an additional hurdle in the preservation strategy is obtained by lowering the water activity. Simultaneously with the weight loss, proteolysis and lipolysis takes place and the amount of free amino acids increases. In addition, anaerobic conditions and the slight acid environment favors the synthesis and activity of decarboxylase enzymes (Bover-Cid *et al.*, 2006). In sausages with a greater diameter the optimal conditions for decarboxylase activity last longer since the anaerobic conditions are more distinct and the salt concentrations remains longer low due to the slower water evaporation. As consequence, big diameter sausages often contain higher biogenic amine levels, especially of TYR, PUT and SPM (Bover-Cid *et al.*, 1999b).

1.4.2 ROLE OF SAUSAGE PROCESSING ON THE NITROSATION OF AMINES

1.4.2.1 INGREDIENTS AND ADDITIVES

Curing salt
Sodium chloride, nitrite and nitrate salts (E 249, KNO_2; E250, $NaNO_2$; E 251, KNO_3 and E252, $NaNO_3$) are the main functional compounds of curing salt. Although curing salt is technologically indispensable in the production of dry fermented sausages, it has a major impact on the *N*-nitrosamine formation. Nitrite develops the typical red cured colour as it reacts with myoglobin to form nitrosomyoglobin, inhibits the growth of pathogenic *Clostridium botulinum* and *Enterobacteriaceae*, and contributes to the characteristic

cured flavour (Toldrà, 2002). Besides the direct addition of nitrite, nitrate can be used in long-term ripened sausages. In that case, nitrate must be converted to nitrite by nitrate reductase positive bacteria such as *Micrococcaceae*. As discussed in Chapter 1.3.1, nitrite can be considered as the main supplier of nitrosating agents. As a consequence, the use of nitrite in meat products is legally defined worldwide. Nowadays in Europe, the restrictions of nitrite is described in directive 2006/52/EC (2006). In general, the directive allows a maximum addition of 150 mg sodium nitrite/kg to all kinds of meat products, plus 150 mg sodium nitrate/kg for unheated meat products like dry cured ham and fermented sausages. Although the current legislation is clear concerning the addition of nitrite and nitrate, it is difficult to determine whether the law is respected by the manufacturers, since nitrite is very reactive and in end products only residual levels can be measured. In that regard, the former legislation was more restrictive. Directive 95/2/EC (1995) recommended the maximum of ingoing amounts (150 mg/kg $NaNO_2$) whereas the residual amounts were limited to 50 mg/kg $NaNO_2$.

Although the nitrite addition is now legally restricted, dangerous high amounts may accidentally be added to meat products, as occurred in the 1930s in Germany. Therefore, nowadays, only premixes of table salt with 0.5-0.6% nitrite can be used in meat preparations (Honikel, 2008). Salt has several technological advantages: (i) inhibiting the growth of contaminating microorganisms and thus limiting biogenic amine accumulation, (ii) increasing the myofibrillar protein solubility and (iii) contributing to the sensorial appreciation of the product (Toldrà, 2002). Moreover, sodium chloride seems to have a slight inhibitory effect on the *N*-nitrosamine formation in meat products. For instance, Rywotycki (2002, 2007) observed the reduction of NDMA and NDEA when more than 2% NaCl was added prior to the pasteurisation of pork ham, while Theiler *et al.* (1981) demonstrated that 1.5% inhibited NPYR formation in minced pork.

Antioxidants

Ascorbic acid (E 300, vitamin C), erythorbic acid (E 315) or their salts (E 301, E 302 and E 316) are used to enhance the colour formation in cured meat. For instance, ascorbic acid will oxidize to dehydroascorbic acid while nitrite is reduced to nitric oxide prior to myoglobin nitrosation. In addition, these antioxidants inhibit the formation of *N*-nitrosamines since they reduce nitrite to nitrogen or nitric oxide and scavenge directly the nitrosonium ion (NO^+). These reactions are faster than the nitrosation of secondary amines (Tannenbaum *et al.*, 1991). Nevertheless, Sebranek (1979) demonstrated that an excess of ascorbic acid (> 2000 mg/kg) added to bacon only reduced the *N*-nitrosamine content with 70%. In fact, the effect of ascorbic acid is limited due to its lack of solubility in adipose tissue. Nitric oxide, which preferentially diffuses to hydrophobic regions, can act as a nitrosating agent in the fat tissue (Sullivan, 2011). In fatty meat products, lipophilic fatty acid esters of ascorbic acid, e.g ascorbyl palmitate (E 304) and propyl gallate (E 310), can inhibit *N*-nitrosamine formation in bacon (Sen *et al.*, 1976). Alternatively, α-tocopherol (E 307, vitamin E) can be added to inhibit the

N-nitrosamine formation in the adipose tissue. In bacon, the addition of α-tocopherol (500 mg/kg) alone or in combination with ascorbate could effectively inhibit the NPYR formation (Fiddler *et al.*, 1978; Reddy *et al.*, 1982). Vitamin E supplementation of the pigs diets is even more preferable. The *N*-nitrosamine inhibition in salami made with the vitamin E enriched meat is more efficient than when α-tocopherol is directly added to the meat batter (Meineri *et al.*, 2013).

Spices and herbs

The most common spices in dry fermented sausages are black pepper, mace and nutmeg, coriander, paprika and chilli pepper (Toldrá, 2007). Despite their contribution to the flavour, some spices can also be a source of *N*-nitrosamines. On the one hand, spices can be sporadically contaminated with *N*-nitrosamines, e.g., NPYR in dried chillies (Tricker, Siddiqi, & Preussmann, 1988) and NDMA in paprika (Atawodi *et al.*, 1993). On the other hand, these spices can also contain precursors of *N*-nitrosamines. For instance, black pepper contains pyrroperine and pyrrolidine as the precursors of NPYR, as well as piperine and PIP which lead to the formation of NPIP (Nakamura *et al.*, 1981). PIP can directly be nitrosated to form NPIP, while the main pungent compound of pepper, piperine (3 – 8 %) (Schulz *et al.*, 2005), is firstly cleaved into piperic acid and PIP. Subsequently, both resulting fractions can be nitrosated to give several unknown C-nitroso compounds and NPIP (Figure 1.15) (Osawa *et al.*, 1982).

During the storage of premixes of spices and nitrite curing salt, the formation of several *N*-nitrosamines was observed (Sen *et al.*, 1974b; Gough & Goodhead, 1975). Therefore, as discussed earlier (section 1.3.3.1) the use of such kind of premixes is banned in the USA. Furthermore, spices might also contribute to the *N*-nitrosamine formation when directly used in nitrite cured meat products, through the addition of precursors. Although many studies associate the formation of NPIP and NPYR in meat products directly with the addition of spices (Yamamoto *et al.*, 1984; Domanska & Kowalski, 2003; Yurchenko & Mölder, 2007), there is no experimental evidence yet.

Besides the risk that spices are involved in the supply of *N*-nitrosamine precursors, plant materials such as spices and herbs can contain antioxidative and nitrite scavenging compounds. Especially plant phenols such as catechol, 4-hydroxychavicol and eugenol, showed inhibitory effects in the nitrosation reaction (Shenoy & Choughuley, 1999). The addition of green tea polyphenols or grape seeds polyphenols (containing e.g., catechin, epicatechin and their gallates) successfully inhibited the *N*-nitrosamine formation during the ripening of dry fermented sausages (Li *et al.*, 2013).

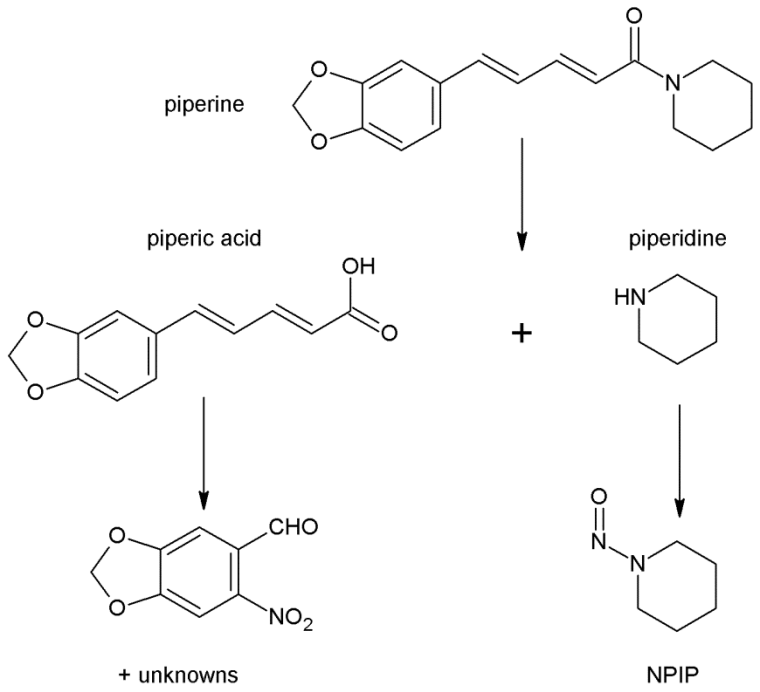


Figure 1.15 Proposed mechanism for the formation of *N*-nitroso compounds formation from the reaction between piperine and nitrite (redrafted from Osawa *et al.*, 1982).

1.4.2.2 PROCESSING CONDITIONS

Fermentation

During the fermentation phase, the starter culture will develop to the dominating flora. As consequence, the LAB will acidify the product. Nonetheless, the final pH of common types of dry fermented sausages (pH 4.2 - 5.5) (Toldrá, 2007) remains far above the optimal pH for nitrosation (pH 2.5 - 3.4) (Bartsch *et al.*, 1988). In regard to the risk of *N*-nitrosamine formation, microorganisms can be responsible for the production of precursors, such as biogenic amines (see Chapter 1.4.1) and nitrite (by reducing nitrate). Moreover, some bacteria were found to produce enzymes which can catalyze the nitrosation of secondary amines (Ayanaba & Alexander, 1973; Thacker & Brooks, 1974; Mills & Alexander, 1976); Kunisaki & Hayashi, 1979). However these strains were of intestinal origin and up till now, no bacteria containing these enzymes have been isolated from dry fermented sausages.

Smoking
Combustion smoke is an important source of nitrogen oxides, which may lead to the release of nitrosating agents (Tricker & Preussmann, 1991). Other compounds of smoke can also influence the nitrosation reaction. As mentioned earlier, smoke contains formaldehyde and acetaldehyde which can lead to the formation of NTHZ and NTCA (Ikins *et al.*, 1988). During the combustion of wood, phenols are formed by the thermal degradation of lignin (Rozum, 2009) and up to 300 mg/kg phenols can be measured in smoked meat. As discussed earlier in this chapter for plant phenols, wood phenols can also inhibit the *N*-nitrosamine formation (Scotter & Castle, 2004).

Ripening and storage
In general, it is assumed that a prolonged ripening time and storage causes a higher *N*-nitrosamine content since the proteolysis of the meat proteins continues. As a consequence free amino acids may be further decarboxylated to biogenic amines. However, depending of the rate of nitrite reduction, the *N*-nitrosamine content will sooner or later reach a maximum level during the ripening period of sausages (Li *et al.*, 2013) or dry cured ham (Wei *et al.*, 2009). Moreover, little to no attention has been paid to the external influences, e.g., decreasing water activity and increasing salt content.

1.5 CONCLUSIONS

Generally, a variety of *N*-nitrosamines can be found in dry fermented sausages. Although these meat products contain mostly low concentrations of these contaminants, it is of great importance to pursue an overall absence because of their carcinogenicity. The last decades great efforts have been made to reduce the *N*-nitrosamine content of meat products, mainly by restricting the added amount of nitrite and the use of nitrite scavenger like ascorbate. Nevertheless, until now, the formation mechanisms in dry fermented sausages are not fully understood. This is mainly because of the complexity of real meat products, e.g. possible precursors, interaction of inhibitors and catalysts, reactivity of nitrite, and other process conditions.

In this work, firstly a reliable and validated method for the determination of biogenic amines in dry fermented sausages was developed (Chapter 2). In Chapter 3, a market survey was performed in order to measure the biogenic amines, residual nitrite and nitrate and *N*-nitrosamine contents. Based on these results, the NPIP formation in dry fermented sausages was studied in detail. Therefore, a dry fermented sausage model was developed (Chapter 4) in order to investigate the influence of CAD and PIP on the NPIP formation (Chapter 5). In Chapter 6, the influence of the water activity and pH change was investigated in a liquid model. Finally, in Chapter 7, the possibility of external contamination by spices was discussed.

CHAPTER 2
ANALYSIS OF BIOGENIC AMINES IN DRY FERMENTED SAUSAGES BY HPLC[2]

[2] This chapter is based on the following paper:
De Mey, E., Drabik-Markiewicz, G., De Maere, H., Peeters, M.-C., Derdelinckx, G., Paelinck, H., & Kowalska, T. (2012). Dabsyl derivatisation as an alternative for dansylation in the detection of biogenic amines in fermented meat products by reversed phase high performance liquid chromatography. Food Chemistry, 130(4), 1017–1023.

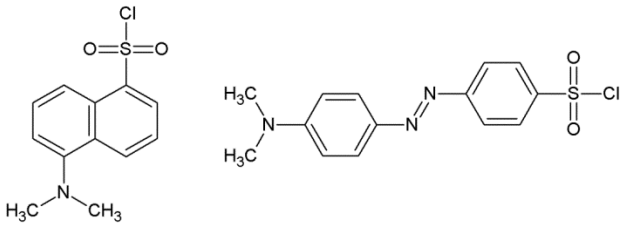
2.1 INTRODUCTION

For quality and safety issues, the monitoring of biogenic amines in dry fermented sausages is important. In the context of this PhD-study, the occurrence of biogenic amines is studied in order to determine their relationship to the formation of *N*-nitrosamines. In the literature, many methods have been described to analyze biogenic amines in several food products. However, to obtain a reliable method, adapted to the available equipment and the target matrix, it is necessary to optimize the analysis.

One considerable difficulty is the isolation of biogenic amines from a complex matrix of the food sample. Solid samples are most frequently extracted with acidic solvents which also act as deproteinisation agents during the liquid-solid extraction (LSE). In meat and fish analyses, trichloro acetic acid (TCA) (Masson *et al.*, 1999; Ferreira, *et al.*, 2006) or, more frequently, perchloric acid ($HClO_4$) (Eerola *et al.*, 1993; Latorre-Moratella *et al.*, 2008; Dadáková *et al.*, 2009) is used. Most research dealing with solid food samples limits the sample clean-up to LSE, although the resulting extracts still contain several interfering compounds. To remove these compounds and concentrate the sample, solid phase extraction (SPE) can be used after the LSE procedure. (Calbiani *et al.*, 2005). However, the difference in the pKa-values of amines makes it difficult to simultaneously elute the target analytes (Molins-Legua & Campins-Falcó, 2005). To enhance the coelution of the target compounds, a C18 cartridge can be used after derivatisation (Soufleros *et al.*, 2007).

Among the available analytical techniques, HPLC is by far the most frequently used to separate and quantify biogenic amines. Since most biogenic amines present in the food samples neither show an adequate light absorption, nor exhibit significant fluorescence, derivatisation has to be performed in order to increase the sensitivity needed for a subsequent UV, VIS or fluorescence detection (Önal, 2007). The use of an HPLC system equipped with an UV detector requires derivatisation with a chromophoric reagent such as dansyl-chloride (Dns-Cl) (Saarinen, 2002; Dadáková *et al.*, 2009), or dabsyl chloride (Dbs-Cl) (Krause *et al.*, 1995; Romero, 2003). Both sulfonyl chlorides are presented in Figure 2.1. Although dansyl chloride is the most widely used derivatisation reagent in the analysis of biogenic amines by means of RP-HPLC-UV, this method demonstrates certain drawbacks, when applied to the determination of biogenic amines in food samples. One drawback is the long derivatisation time, another one is that alkaline ammonia has to be used to remove the interfering by-products.

In this study, the dansylation procedure (Eerola *et al.*1993) is compared with the closely related, yet faster dabsylation procedure. Both derivatization reaction are based on the nucleophilic substitution reaction of the sulfonyl chloride with amine groups.



Dansyl-chloride Dabsyl-chloride

Figure 2.1 Chemical structure of dansyl-chloride and dabsyl-chloride (redrafted from Coppex, 2000).

Although dabsylation is less frequently applied in meat analysis, its advantage over the predominantly used dansyl chloride reagent is that dabsyl derivatives show absorbance in the range of 436 - 460 nm. In that way, interferences from UV-absorbing biological compounds present in the meat extracts are mostly avoided (Aboul-Enein, 2003). For dabsylation of biogenic amines in several foodstuffs, small variations among the methods can be found (Krause *et al.*, 1995; Romero *et al.*, 2000; Castillo & Castells, 2001). Therefore this study is carried out to determine the critical parameters affecting the derivatisation yields of the aforementioned biogenic amines. The ultimate goal is to develop a reliable and robust method for the detection and quantification of several biogenic amines in the meat products.

2.2 EXPERIMENTAL

2.2.1 PREPARATION OF THE AMINE STANDARD SOLUTIONS

The standards (either in the form of the free bases, or the respective hydrochlorides), i.e., SPM, SPD, CAD, PUT, PHE, TYR, SER, HIS and TRYP were purchased from Sigma Aldrich (Bornem, Belgium). A mixed stock solution, containing 1000 µg/mL of each individual amine, was prepared by dissolving adequate amounts of the amines in a mixture of methanol and 1 M HCl (1:1, v/v) (both chemicals purchased from VWR International, Leuven, Belgium). A separate internal standard (IS) stock solution, containing 1000 µg/mL 1,7-diaminoheptane (Sigma Aldrich), was made in an analogous way. These solutions were stored for periods of up to one month at a temperature of -28 °C. Seven working solutions, at concentrations of 0.08, 0.40, 0.80, 2.00, 4.00, 6.00, 8.00 µg/mL of each individual amine, respectively, were obtained from the stock solution by an appropriate dilution with 0.4 M $HClO_4$ (VWR International). These amine mixtures were stored at 4 °C for one week. The IS stock solution was diluted with 0.4 M $HClO_4$ to a concentration of 8 µg/mL, in order to obtain an IS working solution.

2.2.2 LIQUID-SOLID EXTRACTION OF THE MEAT SAMPLE (LSE)

A 2-g aliquot of a finely chopped dry fermented meat sample was spiked with
200 µL of IS working solution. A 10-mL aliquot of 0.4 M $HClO_4$ was added and the mixture was homogenized with an Ultra-Turrax T18 homogenizer (IKA, Staufen, Germany). The extracts were stored at 4 °C to crystallize the fat. The meat sample was centrifuged (Heraeus Labofuge 200, Fisher Scientific, Tournai, Belgium) for 10 min at 1000 g and the upper fat layer was removed. Subsequently, the extraction was repeated. The filtered supernatants were combined and the resulting volume was made up to 25 mL with 0.4 M $HClO_4$.

2.2.3 DERIVATISATION

2.2.3.1 DANSYLATION

A 2-mL aliquot of the amine mixture, or sample extract was transferred to a 10-mL test tube, and the pH was adjusted to 9.5-10.0 by adding 400 µL 2 M NaOH and 600 µL buffer solution (0.95 M $NaHCO_3$). A dansyl chloride solution was freshly prepared each day by ultrasonic dissolution of 10 mg Dns-Cl (1-dimethylamino-naphtalene-5-sulfonyl chloride, Sigma Aldrich) per 1 mL acetonitrile (Fisher Scientific). A 4-mL portion of dansyl chloride solution was added to the test tube and then thoroughly vortexed. Following the procedure elaborated by Eerola *et al.* (1993), derivatisation was carried out for 45 min at 40 °C. After this incubation period, the excess of dansyl chloride was removed by adding a portion of concentrated ammonia (VWR International) and incubating the test tube at room temperature in the dark. In order to obtain a chromatogram without an interference of Dns-Cl, three different volumes (i.e., 0, 100 and 200 µL) of ammonia and two different incubation times (i.e., 30 and 60 min) were tested.

2.2.3.2 DABSYLATION

An analogous volume of the extract as for dansylation (i.e., 2 mL) was used for the derivatisation with dabsyl chloride (4-dimethylaminoazobenzene-4'-sulfonyl chloride, Sigma Aldrich). To study the optimal pH value for dabsylation, portions of 2 M NaOH varying from 150 µL to 500 µL were added to cover the pH range from 7.4 to 10.6. Buffering of the samples was done by adding 600 µL of 0.95 M $NaHCO_3$. The 4-mL portion of the dabsyl chloride solution (5 mg Dbs-Cl per 1 mL acetonitrile) was added to the sample. Incubation was performed at a temperature of 70 °C and 80 °C for 20 min, in order to establish a better dabsylation yield. Ultimately, the reaction was stopped by cooling the test tubes in an ice bath for 30 min.

2.2.4 SAMPLE PURIFICATION AND CONCENTRATION

2.2.4.1 LIQUID-LIQUID EXTRACTION (LLE)

A 2-mL portion of diethyl ether (Fisher Scientific) was added to the derivatised sample and shaken for 1 min. After separation of the two phases, the upper organic layer was transferred to a second test tube, while the lower aqueous layer was re-extracted using another 2-mL portion of diethyl ether. The entire extraction procedure was performed three times. Ultimately, the three organic fractions were combined and evaporated to dryness at 30 °C under a steady stream of nitrogen.

2.2.4.2 SOLID-PHASE EXTRACTION (SPE)

To obtain good recovery of the biogenic amines, an experiment was set up to determine the best possible conditions of carrying out the SPE procedure. Therefore, the different eluted fractions were collected separately and each one was analysed for the target compounds. In the first experiment, SPE purification of the sample was directly applied after the perchloric acid extraction. The Gracepure C18 cartridge (1000 mg/6 mL) (Grace Davison Discovery Sciences, Lokeren, Belgium) was activated with 6 mL acetonitrile and equilibrated with 6 mL ultra-pure water. The extract was loaded on top of the cartridge and washed twice with two 4.5-mL portions of ultra-pure water. Elution of the biogenic amines was forced with three 4.5-mL portions of acetonitrile. In the second experiment, the sample was first derivatised with dabsyl chloride and then treated as in the first experiment. In the third experiment, the C18 cartridge was subsequently activated and conditioned with 6 mL acetonitrile and 6 mL 0.4 M $HClO_4$. The dabsylated extract was loaded on the SPE cartridge and washed with two 4.5-mL portions of 0.4 M $HClO_4$. Finally, the amines were eluted with the three consecutive 4.5-mL portions of acetonitrile. The obtained fractions were concentrated under nitrogen at 35 °C with the use of the TurboVap workstation (Caliper Life Sciences, Teralfene, Belgium).

2.2.4.3 SAMPLE DILUTION

After evaporation to dryness, the residue had to be dissolved in an appropriate solution to prepare the sample for chromatographic analysis. Originally the extract was liquefied in 5 mL mobile phase A (used at the start of the gradient sequence), which was a mixture of methanol, acetonitrile and water (12.5:37.5:50, v/v/v). Then dilution buffers, composed of acetonitrile, ethanol and elution buffer (9 mM NaH_2PO_4) (50:25:25, v/v/v) at the different pH values between 2.0 and 12.5 were made to study the influence on the stability of the extracts.

2.2.5 CHROMATOGRAPHIC PROCEDURE

HPLC was carried out on a Hitachi La ChromElite (VWR International) with UV detector. Prior to use, the two coupled Chromolith®Performance RP-18e columns (100 mm × 3 mm i.d., VWR International) were equilibrated for 24 h with 100% mobile phase A (methanol - acetonitrile – water; 12.5:37.5:50,

v/v/v) and thermostated at 40 °C during the analysis. Aliquots (10-µL) of the derivatised extract were chromatographed at a flow rate of 1.0 mL/min. For the dansylated amines, the following gradient profile was used: from 0 to 25%, mobile phase B (methanol – acetonitrile, 25:75, v/v) in 5 min, 25% for 5 min, an increase to 55% in 6 min, a jump to 100% in 1 min, 100% for 4 min, then a decrease to 0% in 2 min, and finally equilibration at 100% mobile phase A for 3 min. For the less polar dabsyl derivatives, the gradient profile was adjusted, as follows: From 0 to 40%, mobile phase B in 8 min, increase to 60% in 5 min, raise to 100% in 7 min, 100% for 5 min, decrease to 0% in 2 min, and finally equilibration at 100% mobile phase A for 3 min. The detection wavelength was 254 nm for the dansyl derivatives and 450 nm for the dabsyl derivatives.

2.2.6 STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviation (n = 6 or 8). Linear least-square regression was used to calculate the intercepts (a), slopes (b) and the coefficient of determinations (R^2) of the calibration curves (Microsoft Office Excel 2007, Microsoft Corporation, Redmond, WA). To determine the matrix effect, a t-test was performed to compare the slopes of the calibration curves.

2.3. RESULTS AND DISCUSSION

2.3.1 DERIVATISATION

2.3.1.1 DANSYLATION

Although the dansylation procedure - as described by Eerola *et al.* (1993) - is widely used for determination of biogenic amines in all kinds of food samples, its evident drawback is the long derivatisation time. Moreover, ammonia has to be used after the derivatisation in order to remove an excess of the dansyl reagent. Without this step (Figure 2.2 A), the by-products such as dansylamide (Dns-NH_2), dansyl sulphonic acid (Dns-OH) and dansyl hydrazine (Dns-N_2H_3) most likely appear in the chromatogram (marked as * in Figure 2.2). Moreover, an excess of dansyl chloride coelutes with PUT. Unfortunately, an addition of 100 µL concentrated ammonia and incubation for 30 min at room temperature, as suggested by Eerola *et al.* (1993), could not totally remove the excess of dansyl chloride (Figure 2.2 B). As a result, low amounts of the by-products can still be detected in the chromatogram and the peak of PUT does not remain unaffected. Doubling the amount of added ammonia (Figure 2.2 C) or doubling the incubation time did not affect the occurrence of these by-products. Moreover, using the initial gradient as proposed by Eerola *et al.* (1993), the peaks of CAD/ HIS and IS/SER could not be separated. In section 2.3.3 the improvement of the HPLC separation is further discussed.

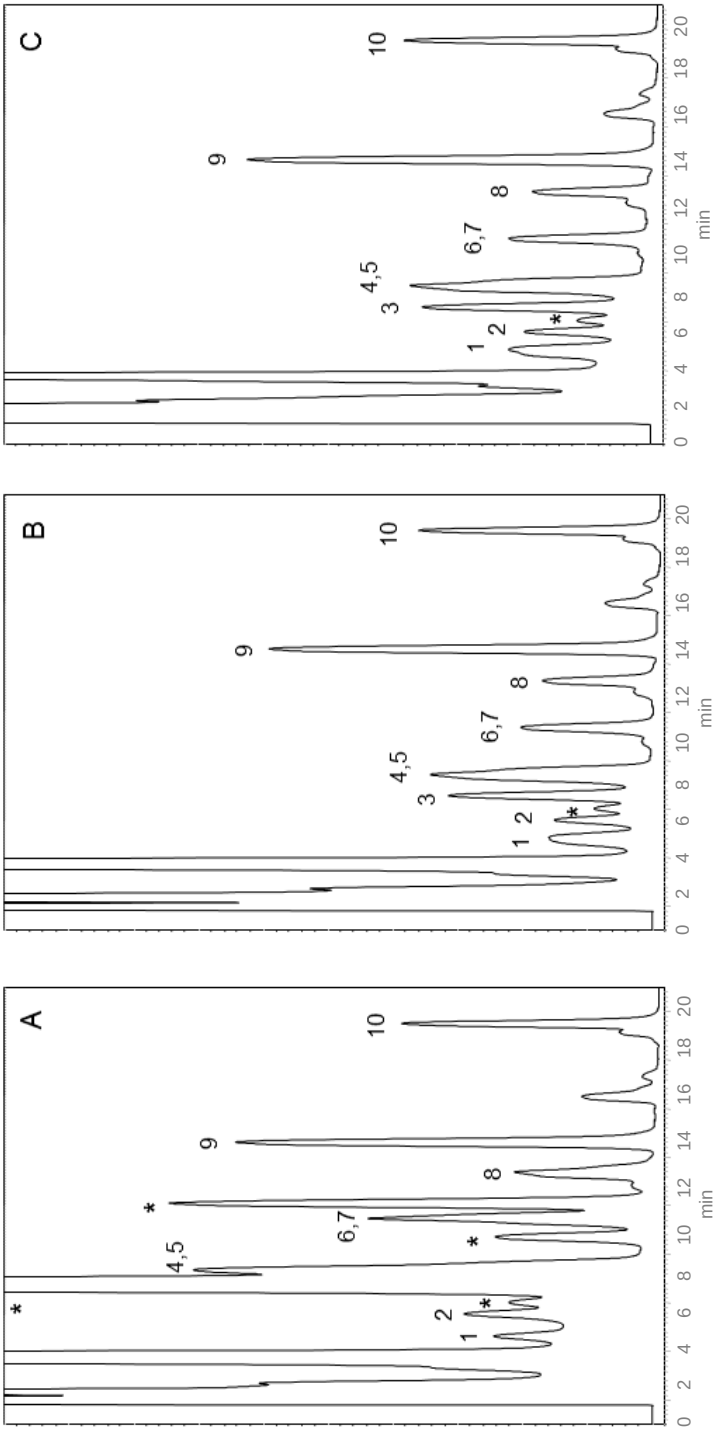


Figure 2.2 Chromatograms of three aliquots of the same spiked dry fermented sausage sample, derivatised with dansyl chloride and the addition of three different volumes of ammonia; i.e., (A) 0 µL, (B) 100 µL, and (C) 200 µL. Identification of the peaks: (1) TYR, (2) PHE, (3) PUT, (4) CAD, (5) HIS, (6) SER, (7) IS (1,7-diaminoheptane), (8) TYR, (9) SPD, (10) SPM.

2.3.1.2 DABSYLATION

In contrast to the dansylation procedure, dabsylation is far less frequently used for the determination of biogenic amines in dry fermented meat products. However, fast dabsylation gives an opportunity to reduce the analysis time. From the literature it appears that the dabsyl derivatisation used to be performed at the temperatures ranging from room temperature to 70 °C. At room temperature, the derivatisation needs an overnight incubation, while at the increased temperatures the incubation time can range between 15 min (Krause *et al.*, 1995) and 30 min only (Chen *et al.*, 2003). In most cases, an incubation period of ca. 20 min at 70 °C is recommended (Romero *et al.*, 2000). In contrast to dansylation (which shows severe decomposition of dansylated amines at temperatures higher than 65 °C), Castillo and Castells (2001) and Dadáková *et al.* (2009) have mentioned that at higher temperatures the peak ascribed to the excess of dabsyl reagent decreased, without the loss of the peak areas of the analytes. In the current study, it was therefore investigated, if dabsylation could be carried out at 80 °C in order to reduce the dabsyl chloride peak. Based on the results obtained, it could be concluded that the derivatisation temperature could not be set at 80 °C without the loss of the analytes (results not shown). The total peak area was by 37% lower, especially due to the reduction of the peaks originating from HIS (96%) and SPM (79%). Although the peak of the excess of dabsyl reagent could not be completely eliminated from the chromatogram, it was ultimately decided to employ 70 °C as an incubation temperature and the test tubes were shaken after 1 and 15 min of incubation, as recommended in the literature (Romero *et al.* 2000). In that way, the highest available yields of the studied analytes were obtained and as the excess peak of the dabsyl reagent was separated from the analytes, no problems were encountered with quantification.

In contrast to the dansylation (pH between 9.5 and 10.0), according Romero *et al*. (2000) the pH range for the dabsyl reaction has to be set between 8.0 and 8.5, with an optimal value of 8.2. Other authors mention buffering of the reaction solution at pH 8.6 (Krause *et al.*, 1995), and even at 9.2 (Castillo & Castells, 2001). To check, if the pH range for the derivatisation is as critical as described by Romero, an investigation was carried out in order to determine the pH range for robust derivatisation. As can be seen from Figure 2.3, derivatisation running under almost neutral pH (e.g., at pH 7.4) has to be avoided. The impact of the pH value on the total peak area and on the peak areas of the individual amines is negligible in the pH range from 8.0 - 10.0. For HIS only, an pH optimum of 8.6 could be detected. Therefore, it was decided to set the pH at ca. 8.6 by adding 300 µL 2 M NaOH and 600 µL buffer solution to the reaction mixture.

Another advantage of using dabsyl chloride is the simple way to remove the excess of reagent. While an addition of irritating ammonia is necessary to remove an excess of dansyl chloride, an easy cooling step, i.e., an ice bath for 30 min, is sufficient to form solid reagent aggregates (Castillo & Castells, 2001).

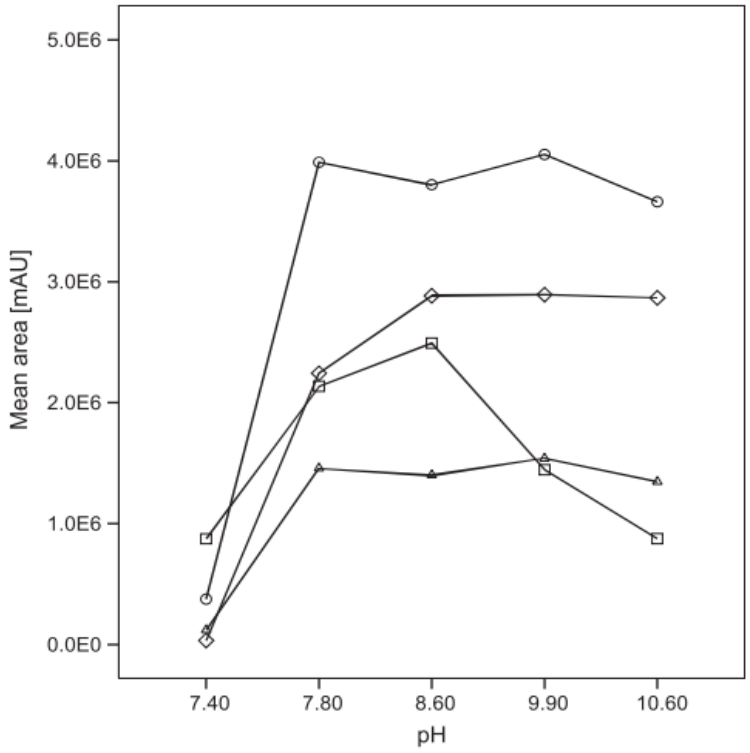


Figure 2.3 Influence of the pH value of the reaction mixture on the dabsyl derivatisation yield at 70 °C with SPD (○), TYR (◊), HIS (□) and SPM (Δ).

2.3.2 SAMPLE PURIFICATION

2.3.2.1 LIQUID-LIQUID EXTRACTION

The method of Eerola *et al.* (1993) does not include additional sample purification, but such steps ought to be considered when analyzing complex food matrices, in particular dry fermented sausage samples. To determine low concentrations of biogenic amines in protein rich matrices, it can be of great importance to remove interfering compounds from the chromatogram with the intention to obtain baseline separated peaks. Therefore an additional LLE with diethyl ether was employed, in order to remove highly polar or ionic compounds from the meat extract. Using three 2-mL portions of diethyl ether was sufficient to extract all biogenic amines. Although the amino acids successfully remained in the aqueous phase, no great improvement of the chromatograms was observed (results not shown). Diethyl ether was probably not selective enough to extract the dansylated amines from the reagent impurities which still interfered in the chromatogram.

2.3.2.2 SOLID PHASE EXTRACTION

The SPE technique was alternatively introduced to provide an extra clean-up. Due to the characteristics of the sample, i.e., high percentage of proteins and ionic compounds, the Gracepure C18-max cartridge with a high carbon percentage (17.1%) was selected. In the course of optimization of the SPE procedure, the different fractions (loading, washing and elution) were separately collected from the cartridge, and their composition was analysed by HPLC in order to examine the distribution of the target compounds in the effluents.

In the first experiment, the SPE purification of the sample was applied directly after the perchloric acid extraction. The cartridge was activated with 6 mL acetonitrile and equilibrated with 6 mL water. It can, however, be seen from Figure 2.4 (A, experiment 1) that the biogenic amines were distributed over all the fractions, due to the great polarity differences of the underivatised biogenic amines. To overcome this problem, the biogenic amines were derivatised prior to the application of the SPE procedure (Soufleros *et al.*, 2007). In that way, the analytes became more polar and were better retained in the course of washing. As shown in Figure 2.4 (A, experiment 2), after modification of the original procedure, the greater part of the biogenic amines was collected in the elution fractions. However, a small percentage was still eluted in the loading and the first washing fraction. The main reason was an inadequate retention of PHE (Figure 2.4, B) and TRYP (data not shown). Finally, the retention of all the amines during the washing phase was guaranteed when water (used as the equilibration/washing solvent) was replaced with 0.4 M $HClO_4$ (Figure 2.4, B, experiment 3). Moreover, addition of 0.4 M $HClO_4$ improved the elution and only one 4.5-mL portions of acetonitrile was enough to elute all biogenic amines. Similar to the extraction with diethyl ether, acetonitrile as elution solvent was evaporated to concentrate the extract.

2.3.2.3 SAMPLE DILUTION

Dissolution of the dry residue was first made in 5 mL mobile phase A (methanol – acetonitrile - water, 12.5:37.5:50, v/v/v), but based on the area ratio HIS/IS, HIS seemed unstable at room temperature, when kept for 24 h. Krause *et al.* (1995) suggested using a dilution buffer to overcome the crystallization problem of certain biogenic amines (such as HIS and TYR) during a prolonged storage time. This recommended dilution buffer was composed of acetonitrile, ethanol and elution buffer (9 mM NaH_2PO_4) (50:25:25, v/v/v). The use of this solution (buffered within the pH range from 2.0 to 12.5) showed that the stability of HIS could be improved, when the extract was buffered at ca. pH 7 (Figure 2.5). However, the introduction of the dilution buffer – regardless of the applied pH value – induced instability of SPD and the yield of SPM could even be reduced to zero. Therefore it was finally decided to keep the mobile phase A as the dry residue solvent. The stability of HIS could be ensured by keeping the HPLC vials at 4 °C until analysis time. With this precaution, the derivatives in the extracts showed less than 1% degradation within a period of 12 h.

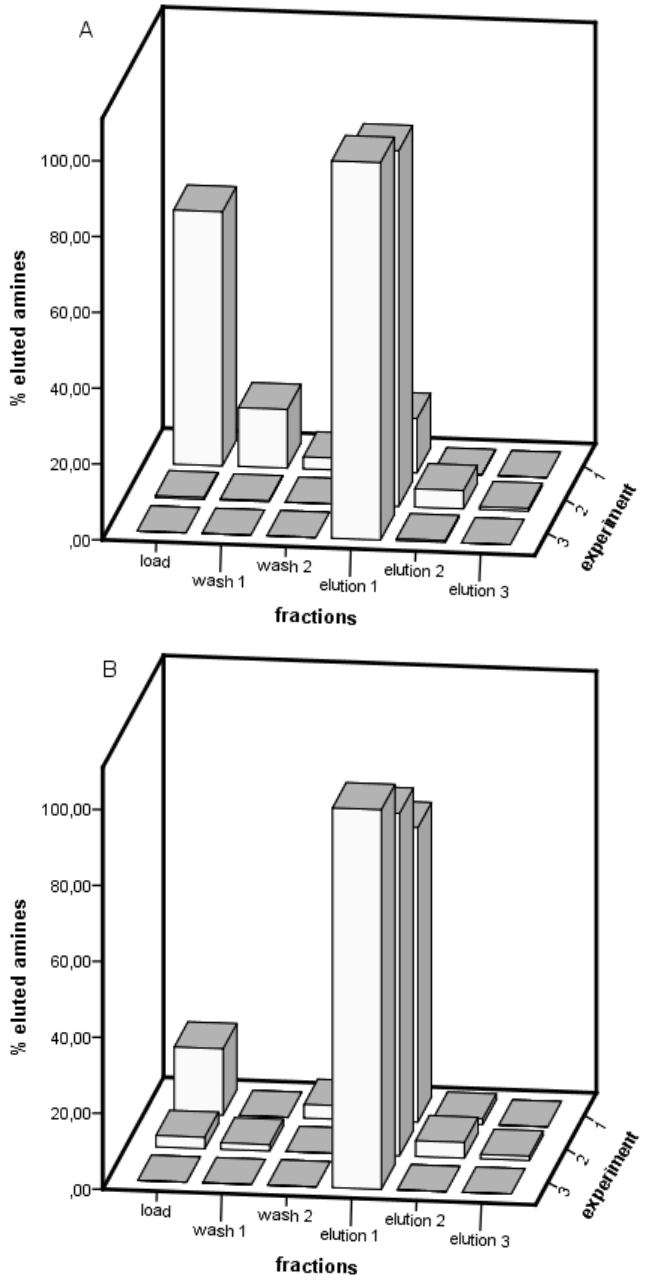


Figure 2.4 Distribution of the target amines; (A) total biogenic amines, and (B) PHE among the different fractions collected during the SPE purification; experiment 1: 6 mL underivatised extract, washed with 2 x 4.5 mL water and eluted with 3 x 4.5 mL acetonitrile, experiment 2: 6 mL dabsylated extract, washed with 2 x 4.5 mL water and eluted with 3 x 4.5 mL acetonitrile, experiment 3: 6 mL dabsylated extract, washed with 2 x 4.5 mL 0.4 M $HClO_4$ and eluted with 3 x 4.5 mL acetonitrile.

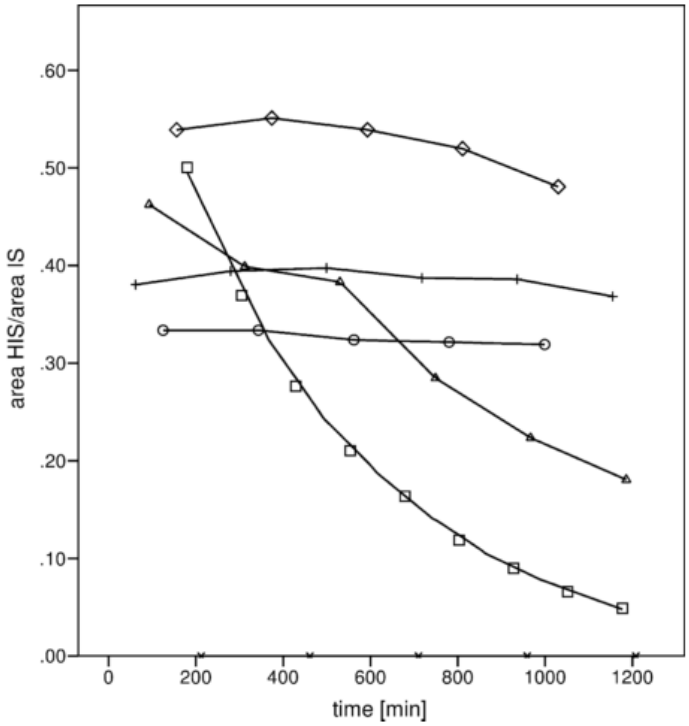


Figure 2.5. Degradation of dabsyl-HIS (area HIS/area IS) in time, buffered in a diluting solution (acetonitrile – ethanol – dilution buffer (9 mM NaH_2PO_4), 50:25:25, v/v/v) and adjusted with 2 M NaOH at pH 2.0 (□), pH 3.7 (Δ), pH 4.0 (○), pH 7.3 (◊), pH 7.6 (+) and pH 12.5 (×).

2.3.3 HPLC SEPARATION

For the separation of dansylated biogenic amines, a simple gradient program, as proposed by Eerola *et al.* (1993), was initially tested. The mobile phase consisted of 0.1 M ammonium acetate and acetonitrile (50:50) and was linearly converted to a ratio of 10:90. Two Chromolith columns were coupled, so the retention times of the biogenic amines were prolonged and they could be separated from the more polar peaks. However, in order to separate the CAD/HIS and IS/SER pairs, methanol as an organic modifier was added to the acetonitrile (25:75 v/v). This change improved the selectivity of both coeluting pairs of compounds. Furthermore, ammonium acetate was eliminated, as it exerted no visible positive effect on the reduction of the peak tailing. Thus, the 0.1 M ammonium acetate was replaced by ultra-pure water. The final gradient employed for the separation of dansylated biogenic amines is described in section 2.2.5.

Because dabsylated biogenic amines are more hydrophobic than the dansylated compounds, the gradient proposed for the separation of dansylated amines had to be slightly modified. For the elution of the most

hydrophobic compounds (such as SPD and SPM) a faster increase in solvent strength was recommended. The gradient used for the separation of dabsylated biogenic amines is given in section 2.2.5.

Although the total analysis time of the dabsylated amines is by 5 minutes longer than that for the dansylated compounds, this difference is repaid by the advantage of obtaining an interference-free chromatogram. In the case of dansylated amines, PUT could not be separated from the excess reagent and the impurities, and its quantification was unfeasible. In general, the derivatisation reagent type does not influence the elution order of the biogenic amines, except for SER and 1,7-diaminoheptane (IS). In the case of dabsylation SER elutes just before the IS (Figure 2.6), while in the case of dansylation it leaves the column after the IS (chromatogram not shown).

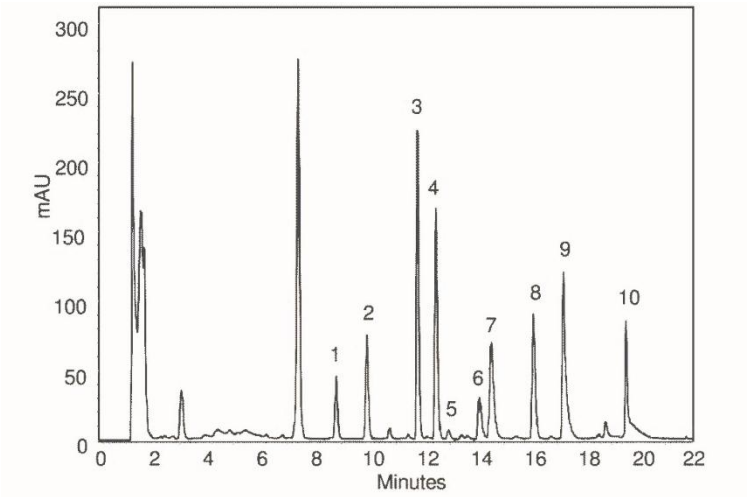


Figure 2.6 Chromatogram of dabsylated amines extracted from a dry fermented sausage sample spiked at a concentration of 100 mg/kg meat. Numbers as in Figure 2.2.

2.3.4 METHOD VALIDATION

The newly elaborated method which includes dabsyl derivatisation and the optimised SPE purification (Figure 2.7) underwent a validation procedure, in order to make it possible to use this method in the future routine analysis of food samples (such as dry fermented sausages).

2.3.4.1 LINEARITY, RANGE AND INSTRUMENTAL DETECTION LIMIT

Standard calibration curves (n = 7) were made by plotting the relative responses (area standard/area IS) versus the concentration of each biogenic amine standard. This was done in triplicate. For all standard curves, $R^2 \geq 0.998$ was observed. The instrumental detection limit was estimated as the concentration of the standard solution at the level of the signal-to-noise ratio equal to three (LOD: S/N = 3). For PHE, PUT, CAD, TYR, SPD and SPM,

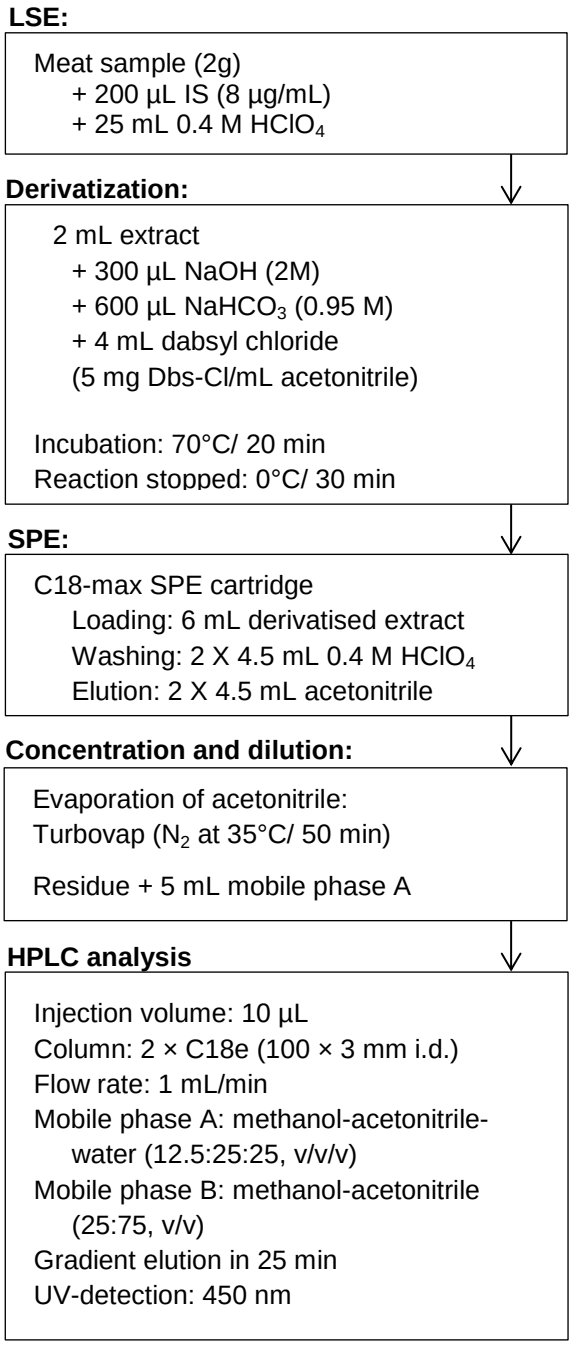


Figure 2.7 Schematic overview of the optimised method, using dabsyl derivatisation and SPE purification.

the LOD level was lower than 0.008 µg/mL and for TRYP, SER and HIS, the LOD level was ca. 0.040 µg/mL.

2.3.4.2 MATRIX EFFECT

To check the matrix effect, a calibration curve in the meat matrix was elaborated. Therefore the fermented meat samples were spiked with working solutions of the biogenic amine standards at concentration levels of 1, 2, 5, 10, 25, 50, 75 and 100 mg/kg meat. This was done in triplicate. A t-test was performed to confirm the equality of the slopes of the standard calibration curve and the calibration curve developed in the meat matrix (Loco, 2006). As the slopes of the calibration curves in the meat matrix did not significantly differ from those obtained for the standard calibration curves. Thus, it can be concluded that the standard calibration curves can be used for quantification of the target compounds in the meat samples.

2.3.4.3 METHOD DETECTION LIMIT AND QUANTITATION LIMIT

The method detection limit (MDL) was estimated by spiking eight dry fermented meat samples at a concentration of 1 mg/kg meat. The samples were subjected to the full analytical procedure. Because no blank meat samples were available, the non-spiked meat samples were analysed as the blanks, in order to subtract the respective values from the data valid for the spiked samples. The MDL can be calculated as $t(n_{-1,1-\infty} = 0.99) \times$ standard deviation, where the Student's t-value for the 8 samples at the 99% confidence level was 2.998. Subsequently, samples (n = 8) were spiked with increasing concentrations, which were on average 3 times higher than the determined MDL level. The lowest concentration of which the precision was still acceptable (10% relative standard deviation (RSD)) was considered as the method quantitation limit (MQL). The MDL and MQL-values of the target compounds are given in Table 2.1.

2.3.4.4 ACCURACY AND PRECISION

Dry fermented sausage samples (n = 6) were spiked at two levels, i.e., at the 50 and 100 mg/kg meat level, in order to evaluate the accuracy (expressed as recovery) and precision (repeatability) of the analytical procedure. The RSD was used to express the precision, and the recovery (T%) was calculated. In Table 2.1, the recovery and repeatability data for all biogenic amines are given. The RSD for each biogenic amine determined in dry fermented meat samples was lower than 5%.

Table 2.1 Repeatability (r) expressed as RSD (%) and the recovery (T%, mean ± standard deviation) for dry fermented sausage samples spiked at two levels (50 and 100 mg/kg meat, n=8), the method detection limit (MDL, mg/kg meat) and method quantitation

Dry fermented sausage		Addition level I: 50 mg/kg meat			Addition level II: 100 mg/kg meat			MDL	MQL
	Initial content (mg/kg)	Content after addition (mg/kg)	RSD (%)	T (%)	Content after addition (mg/kg)	RSD (%)	T (%)		
TRYP	2.1	53.2	1.9	102.2 ± 2.2	99.8	1.6	97.7 ± 1.6	0.9	3.2
PHE	nd	44.2	2.3	88.5 ± 2.2	89.9	3.3	89.9 ± 3.0	0.2	0.4
PUT	0.9	47.3	3.3	92.9 ± 3.4	95.4	2.1	94.5 ± 2.0	0.2	0.4
CAD	0.6	48.1	2.3	95.0 ± 2.4	98.7	2.0	96.5 ± 1.9	0.1	0.3
HIS	nd	43.0	3.2	86.0 ± 3.4	88.5	3.8	88.5 ± 3.6	0.9	3.1
SER	nd	43.0	4.3	86.0 ± 4.1	87.2	3.1	87.2 ± 2.7	0.2	0.5
TYR	nd	48.3	4.0	96.5 ± 4.2	94.1	3.9	94.1 ± 3.6	1.0	3.2
SPD	1.1	47.5	3.5	93.0 ± 3.7	90.7	3.5	89.6 ± 3.2	0.2	0.6
SPM	1.5	49.8	3.1	96.6 ± 3.4	90.3	3.5	88.7 ± 3.2	0.9	2.9

2.4. CONCLUSIONS

In dry fermented meat analysis, the most commonly applied HPLC-based method to determine biogenic amines includes a dansylation step. However, this method suffers from selectivity problems due to the presence of an excess reagent and the impurity peaks. Therefore the use of highly irritating ammonia is unavoidable, but even this procedure cannot remove the interfering peak completely, which makes quantification of PUT unfeasible. Contrary to that, the dabsylation can be performed without any use of ammonia or other scavenging reagent. Moreover, the derivatisation temperature in the dabsylation procedure can be raised to 70 °C, without any loss of the analytes. As a result, the reaction time can be reduced to 20 minutes instead of the 45-minute period with dansylation running at 40 °C.

To ensure the baseline separation, the feasibility of an extra purification step was explored. The use of a classical LLE method with diethyl ether as an extraction solvent proved insufficient. Instead, a C18-SPE clean-up step of the derivatised extract was devised, which resulted in a repeatable procedure. This procedure included an acid-founded (0.4 M $HClO_4$) washing step and an elution with an organic solvent (acetonitrile). The sensitivity was improved by the possibility to concentrate the sample in absence of interfering compounds.

Thus in order to develop a robust method to separate and quantify biogenic amines (with the most sensitive histamine as a critical biogenic amine case), one has to dissolve the extract residue in the solvent mixture identical to the mobile phase A and to store the obtained solution under refrigeration until the time of analysis. Application of buffering solutions (9 mM NaH_2PO_4) to prevent crystallization of the amines was discarded, since addition of the buffer negatively affected the stability of the natural polyamines SPD and SPM.

Finally, the alternative method combining dabsyl derivatisation and SPE purification successfully passed the validation procedure. It is confirmed that with the use of this method, the 9 investigated biogenic amines originating from a complex matrix such as dry fermented meat, can easily be identified and reliably quantified.

CHAPTER 3
A SURVEY OF COMMERCIAL DRY FERMENTED SAUSAGES FOR *N*-NITROSAMINES, BIOGENIC AMINES AND RESIDUAL NITRITE[3]

[3] This chapter is based on the following paper:
De Mey, E., De Klerck, K., De Maere, H.; Dewulf, L., Derdelinckx, G., Peeters, M.-C., Fraeye, I., Vander Heyden, Y., Paelinck, H. (2014). The occurrence of *N*-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. Meat Science, 96(2), 821–828.

3.1 INTRODUCTION

On the Belgian market, a diverse range of dry fermented sausages can be found. For instance, the variety in process parameters and meat batter formulations, such as the fresh meat origin and used spices, is endless. In Europe a distinction can roughly be made between Northern and Southern types of fermented sausages. On the one hand, Mediterranean products (Southern types) are characterized by a long ripening time, which results in a low water activity ($a_w < 0.90$) and a rich natural flavour. These products, e.g., Italian Milano Salami and French Rosette, are often covered with molds which contribute to the specific flavour, due to proteolysis. On the other hand, Northern dry fermented sausages have a distinctive acidification step and a shorter drying period, resulting in a final pH between 4.5 and 5.0, and higher water activity ($a_w > 0.90$). Instead of covering the surface with molds, the surface of these sausages is smoked, e.g., Dutch Boerenmetworst and German Cervelatwurst, to prevent undesired yeast growth. In most cases pork meat is used, while combinations of pork and beef can be found in types like Salami d'Ardennes and horse meat in Boulogne. Furthermore, for religious considerations turkey is used to provide halal products. Besides the application of different process parameters and/or meats to obtain a characteristic taste, extraordinary seasonings, for instance, sweet paprika in Spanish Chorizo, or excessive amounts of spices, like black pepper in pepper salami, are often added (Toldrá, 2007).

In this chapter the safety of dry fermented sausages available on the Belgian market, regarding the occurrence of nitrite, nitrate, biogenic amines and volatile *N*-nitrosamines was examined. In addition, the data was explored by multivariate methods, more specifically principal component analysis (PCA) and hierarchical cluster analysis (HCA) in order to search for patterns in the occurrence of biogenic amines and *N*-nitrosamines in the commercial meat products, and their relation with physical and chemical characteristics.

3.2 MATERIAL AND METHODS

3.2.1 SAMPLES

A total of 101 dry fermented sausages were collected in spring 2011 representing the current market supply of 4 major food stores in Belgium. In stores 1 and 2, classified as supermarkets, promoting A-brands, 38 and 33 samples, respectively, were purchased. In stores 3 and 4, two discount stores selling low-budget products, the range was restricted to 22 and 8 products, respectively. The dry fermented sausages were categorized by label information. The products were stored at 7 °C until the end of shelf life. After the storage period, the whole sample was minced and homogenized in a 2096 Homogenizer (FOSS, Hillerød, Denmark) and divided into portions to perform the analyses.

3.2.2 PHYSICAL AND CHEMICAL ANALYSES

The pH of the minced sausage samples was measured by inserting the glass pH electrode in the meat portion (Knick Portamess, Elscolab, Terschuur, The Netherlands). The water activity (a_w) was determined using a dewpoint hygrometer (Aqualab, Decagon Devices, Pullman, USA). Moisture content was determined by drying a homogenized test portion to constant mass at 103 °C (ISO 1442, 1997). Subsequently the dried test portion was extracted with n-hexane in order to determine the free fat content (ISO 1444, 1996). The protein content was estimated from the amount of nitrogen measured by the Kjeldahl method (ISO 937, 1978). Salt content, expressed as g NaCl/100 g product, was determined by the argentometric method (Gros *et al.*, 2005) using an automatic titrator (Titrino, Metrohm, Herisau, Switzerland). All reported values represent the mean of three random measurements of the sausage sample.

3.2.3 CHROMATOGRAPHIC ANALYSES

3.2.3.1 RESIDUAL NITRITE AND NITRATE DETERMINATION

Aliquots (1 g) of the dry fermented sausage samples were extracted by adding 10 mL hot ultra-pure water followed by incubation for 15 min in a hot water bath (ED-19, Julabo, Allentown, USA) at 55 °C. Subsequently, the mixture was centrifuged (Heraeus Labofuge 200, Thermo Fisher Scientific, Waltham, USA) for 10 min at 1000 g. The supernatant was deproteinised with 5 mL acetonitrile (HPLC grade, Thermo Fisher Scientific) and incubated at 4 °C to solidify the fat for easy removal. The extracts were filtered through an Acrodisc syringe filter GxF/Glass (∅ 25 mm, Pall, Zaventem, Belgium) and the volume adjusted to 25.0 mL with ultra-pure water. After filtration through a 0.2 µm filter (Acrodisc GHP), 20 µl was injected onto the HPLC-UV (La Chrom Elite, VWR-Hitachi, Darmstadt, Germany). Nitrite and nitrate were separated by ion chromatography using a Hamilton PRP-X100 column (150 mm, 4.6 mm i.d., Grace Davison Discovery Sciences, Lokeren, Belgium) and isocratic elution with NaCl/NaH_2PO_4 (40 mM/ 2mM) at a flow rate of 2.5 mL/min. The detection of the ions was carried out at 210 nm. The nitrite and nitrate levels were expressed as residual $NaNO_2$ and $NaNO_3$, respectively. The MDL and MQL values of both compounds are given in Table 3.1.

Table 3.1 Method detection limits (MDLs) and method quantitation limits (MQLs) of nitrite and nitrate.

Nitrosating reagens	MDL (mg/kg)	MQL (mg/kg)
$NaNO_2$	1.0	5.0
$NaNO_3$	1.0	5.0

3.2.3.2 BIOGENIC AMINE DETERMINATION

The biogenic amines were analysed as described in Chapter 2 and schematically presented in Figure 2.7.

3.2.3.3 *N*-NITROSAMINE DETERMINATION

The volatile *N*-nitrosamines were analysed according to the method of Drabik-Markiewicz *et al.* (2009). Meat samples (50 g) were spiked with 10 µg/kg NDPA (Sigma Aldrich) and mixed with 200 mL 3 M KOH (VWR International). The volatile *N*-nitrosamines were then extracted from the meat samples by means of vacuum distillation (Heidolph Laborota 4010-digital, Schwabach, Germany). After vacuum distillation, the extraction method of Gasarasi (2001), with some modifications was carried out. Firstly, the distillate (150 mL) was mixed with 4 mL 37% HCl (VWR International, West Chester, PA, USA) and extracted three times with 50 mL of dichloromethane (DCM, Merck, Darmstadt, Germany). Secondary, liquid–liquid extraction was performed on the DCM fraction with 2×50mL 6 M HCl. After drying the extract with anhydrous sodium sulfate (VWR International), the filtrate was concentrated to 100 µL in a Kuderna-Danish apparatus (Sigma Aldrich) in a hot water bath of 65 °C. For the detection and quantification of *N*-nitrosamines, i.e., NDMA, NDEA, NDBA, NPIP, NPYR and NMOR, a GC-TEA (Thermo Electron Cooperation) was used. The extracts (5 µl) were injected on a packed column (10 % Carbowax 20 M + 2% KOH on Chromosorb WAW, 80/100 mesh, 1.8 m, 2 mm i.d. Varian, Middelburg, The Netherlands) and a chromatographic separation was carried out by using argon as carrier gas (25 mL/min). The injection port was set at 175 °C and the oven temperature was increased from 110 °C to 180 °C at 5 °C/min. The temperatures of the interface and pyrolizer of the TEA were set at 250 °C and 500 °C, respectively. The MDL and MQL values of the analysed *N*-nitrosamines are included in Table 3.2.

Table 3.2 Method detection limits (MDLs) and method quantitation limits (MQLs) of the chromatographically determined *N*-nitrosamines.

N-nitrosamines	MDL (µg/kg)	MQL (µg/kg)
NDMA	0.2	0.8
NDEA	0.3	0.9
NDBA	0.6	2.0
NPIP	0.8	2.5
NPYR	0.5	1.6
NMOR	0.5	1.5

3.2.4 STATISTICAL EVALUATION

Statistical calculations (median, minima and maxima,…) were done by PASW Statistics 18.0.0 (SPSS, Chicago, USA). For the exploratory data analysis, subroutines developed under Matlab 5.3 software (The MathWorks, Natick, MA, USA) were used to perform PCA and HCA. Prior to PCA and HCA, the data matrix variables were pretreated with autoscaling.

3.3 RESULTS AND DISCUSSION

3.3.1 PHYSICAL AND CHEMICAL COMPOSITION OF THE RETAIL PRODUCTS

The dry fermented sausages available on the Belgian market were divided in different categories. Based on the label information, the products were categorized according to the regional origin and the use of certain meat species or dominant spices in the recipes. In this way, 15 categories of dry fermented sausage products were obtained. Of the 101 samples, 52 can be considered as South European types while the other 49 were produced as Northern types. In stores 1 and 2, the A-brand stores, the largest range of products (38 and 33, resp.) can be found and almost every sausage category is represented by several trademarks. In the discount stores, the available assortment is smaller, 22 and 8 products for stores 3 and 4, respectively. Because the offered selection is limited, not all categories were present and mainly northern products, e.g., Boulogne, Boerenring and Garlic Salami, were not available in the discount stores.

The results of the physical and chemical analyses of the sausages are reported in Table 3.3. The lowest pH (pH 4.4) was recorded for Rosette salami (French type). The ready-to-eat Snack sausages showed the highest value (pH 6.7). Dry sausages and Italian types, like Milano, did not have a clear acidification due to the missing lactic acid producing starter culture. To obtain shelf stable products, the pH must be lower than 5.2 and the water activity must be reduced below 0.95 (Toldrá, 2007). To reduce the spoilage of mildly fermented sausages (pH > 5.2), the water activity of the commercial products must be lower than 0.91. The small sized Snack sausages are strongly dried (a_w 0.71) resulting in a higher salt concentration (4.5 g/100g) and decreased moisture protein ratio. In this study, the median salt concentration is 3.6 g/100g. The current trend is to reduce the salt content (more specific the sodium content) to obtain healthier meat products. As a result, salt concentrations as low as 2.5 g NaCl/100g, can be found in the products available on the Belgian market.

The median moisture content of the investigated sausages is 40.7 g/100g with a large range, depending on the drying rate. Moisture contents of 11.6 g/100g in Snack sausage up to 55.3 g/100g in Turkey salami were found. Due to the partial substitution of fat by water in the Light salami, the final fat content of this kind of products is only 17.3 g/100g. The median fat content of the Belgian retail products was 29.6 g/100g. The maximum fat (44.1 g/100g) and protein contents (36.0 g/100g) were found in Snack sausages.

Table 3.3 Physical and chemical composition of the commercial dry fermented sausages, the results are presented as median (min-max) values.

		Physical analysis		Chemical analysis (g/100g)			
Type	n	pH	a_w	Moisture	Free fat	Protein	NaCl
North European types							
Boerenring (No. 1)	3	5.0 (4.8-5.0)	0.92 (0.88-0.93)	38.8 (32.4-40.6)	31.5 (26.3-36.6)	20.2 (19.8-21.0)	3.4 (3.0-3.7)
Boulogne (No. 2)	3	4.7 (4.5-4.7)	0.93 (0.88-0.94)	43.2 (40.0-43.7)	28.7 (28.7-38.8)	20.7 (18.8-20.7)	3.5 (2.9-3.6)
Garlic salami (No. 6)	6	4.8 (4.7-4.9)	0.94 (0.92-0.95)	42.3 (38.8-46.3)	31.2 (28.8-35.6)	19.4 (16.3-19.5)	3.0 (2.6-3.4)
German types (No. 7)	2	4.8 (4.6-5.0)	0.94 (0.94-0.94)	44.8 (43.0-46.7)	30.7 (27.3-34.2)	16.5 (15.6-17.4)	3.0 (2.9-3.0)
Light salami (No. 9)	5	4.9 (4.8-5.0)	0.94 (0.87-0.92)	48.3 (45.6-55.3)	17.3 (13.6-22.3)	23.1 (20.4-28.1)	3.2 (2.6-3.6)
Pepper salami (No. 10)	4	4.9 (4.7-5.0)	0.93 (0.91-0.93)	39.7 (33.5-49.7)	29.7 (23.3-35.6)	19.9 (18.5-22.2)	3.3 (2.8-3.5)
Salami (No. 11)	10	5.0 (4.6-5.6)	0.91 (0.88-0.94)	41.7 (37.1-48.5)	30.3 (22.6-40.0)	21.7 (16.8-24.7)	3.7 (2.5-4.3)
Smoked salami (No. 13)	10	4.9 (4.5-5.1)	0.94 (0.91-0.95)	46.0 (40.0-49.3)	28.9 (23.6-36.1)	18.5 (17.2-25.1)	3.2 (2.5-4.0)
Turkey salami (No. 15)	6	4.7 (4.6-5.1)	0.93 (0.91-0.95)	50.1 (42.2-57.2)	21.7 (14.7-28.2)	22.1 (19.5-23.4)	3.7 (3.4-4.1)
South European types							
Chorizo (No. 3)	10	4.8 (4.6-5.6)	0.90 (0.91-0.94)	41.0 (24.3-50.6)	29.5 (19.7-39.0)	23.9 (18.5-27.6)	3.7 (3.1-4.5)
Dry sausage (No. 4)	6	5.3 (5.2-6.2)	0.88 (0.83-0.89)	34.0 (30.4-39.0)	29.4 (25.8-32.9)	26.0 (23.4-31.1)	4.3 (4.0-5.0)
French types (No. 5)	6	5.0 (4.4-5.5)	0.90 (0.89-0.96)	41.4 (37.6-53.8)	24.8 (18.5-31.8)	23.7 (21.3-27.5)	3.9 (2.6-4.3)
Italian types (No. 8)	9	5.5 (5.1-5.9)	0.91 (0.87-0.92)	41.0 (32.5-45.3)	27.9 (20.2-30.9)	24.9 (22.1-27.0)	3.7 (3.5-4.2)
Saucisson d'Ardennes (No. 12)	6	4.9 (4.8-5.6)	0.91 (0.84-0.93)	40.4 (36.5-46.5)	31.5 (17.9-34.2)	22.1 (19.7-29.8)	3.5 (3.1-5.3)
Snack (No. 14)	15	5.6 (5.0-6.7)[b]	0.71 (0.66-0.86)	18.0 (11.6-29.2)	36.7 (25.2-44.1)	30.9 (23.7-36.0)	4.5 (3.5-5.1)
Total	101	5.0 (4.4-6.7)	0.91 (0.66-0.96)	40.7 (11.6-57.2)	29.6 (13.6-44.1)	22.4 (15.6-36.0)	3.6 (2.5-5.3)

3.3.2 RESIDUAL NITRITE AND NITRATE CONTENT

The screening of the commercial sausages at the end of shelf life revealed median residual sodium nitrite and sodium nitrate levels of 6.3 and 11.8 mg/kg, respectively (Table 3.4). A large variance in nitrite and nitrate levels is seen; ranging from not detected to 147.5 mg $NaNO_2$/kg and 167.8 mg $NaNO_3$/kg. The most recent directive (Directive 2006/52/EC, 2006) imposes that the initial addition of sodium nitrite and nitrate in non-heated meat products is restricted to 150 mg/kg each. It is known that a strong reduction of nitrite and nitrate takes place during the processing. As such, the measured residual amounts in the end products do not necessarily reflect the initially added amounts. Consequently, it is impossible to verify the correct application of the current directive by analyzing the end products. The former directive (Directive 95/2/EC, 1995) was more enforceable, since a limit for the residual sodium nitrite was also included. In this study 87% of the analysed samples contained residual sodium nitrite levels under that former limit of 50 mg $NaNO_2$/kg.

Table 3.4 Residual sodium nitrite and sodium nitrate concentrations in commercial dry fermented sausages, the results are presented as the median (min-max) concentration (mg/kg).

Type	n	$NaNO_2$[a,b]	$NaNO_3$[a,b]
North European type			
Boerenring (No. 1)	3	nd	38.2 (25.1 - 100.7)
Boulogne (No. 2)	3	11.4 (nd - 62.5)	47.6 (23.0 - 159.5)
Garlic salami (No. 6)	6	9.5 (nd - 52.9)	20.9 (nd - 130.7)
German types (No. 7)	2	20.9 (5.9 - 35.9)	16.5 (12.4 - 20.6)
Light salami (No. 9)	5	6.4 (nd - 13.9)	nd (nd - 107.3)
Pepper salami (No. 10)	4	nd (nd - 10.5)	70.2 (*4.2* - 158.9)
Salami (No. 11)	10	5.5 (nd - 81.8)	13.7 (nd - 167.8)
Smoked salami (No. 13)	10	nd (nd - 9.2)	11.5 (nd - 130.7)
Turkey salami (No. 15)	6	6.5 (nd - 12.3)	20.6 (nd - 94.0)
South European type			
Chorizo (No. 3)	10	8.7 (nd - 147.5)	11.3 (nd - 73.8)
Dry sausage (No. 4)	6	nd (nd - 7.7)	9.4 (nd - 20.4)
French types (No. 5)	6	*2.4* (nd - 108.0)	9.1 (nd - 105.3)
Italian types (No. 8)	9	32.4 (nd - 82.2)	nd (nd - 24.3)
Saucisson d'Ardennes (No. 12)	6	5.5 (nd - 74.5)	20.8 (nd - 55.2)
Snack (No. 14)	15	16.5 (nd - 95.9)	11.8 (nd - 60.3)
Total	101	6.3 (nd – 147.5)	11.8 (nd – 167.8)

[a] nd: not detected, values below the MDL.
[b] values estimated below the MQL are printed in italics

3.3.3 BIOGENIC AMINES ACCUMULATION

In general the accumulation of biogenic amines at the end of shelf life was low and a large variability in the nature and quantity of the available biogenic amines was found in the different types of dry fermented sausages (Table 3.5). Traces of SER were only detected in Snack sausage samples which contain walnuts, a rich source of SER (Feldman & Lee, 1985). In 77% of the samples no HIS was detected. In a few cases the concentrations were notably higher. For example, in some Turkey salami samples the HIS content exceeded the maximum concentration of 100 mg/kg as allowed in scombroid fish (Commission regulation (EC) No 2073/2005). No specific legislation exists in Europe about the HIS content in meat products. Besides SER and HIS, low levels of TRYP and PHE were measured in the dry fermented sausage samples. These are probably due to the non-specific activity of phenylalanine decarboxylase in micrococci and staphylococci, which are able to grow during the beginning of the fermentation process (Nakazawa *et al.*, 1977).

This study confirms that TYR is the major biogenic amine in dry fermented sausages (Latorre-Moratella *et al.*, 2008). Concentrations ranged from not detected to a maximum of 411 mg/kg. It is recommended to susceptible individuals to avoid TYR rich foods, as it can cause migraine, mainly when drugs e.g., MAOIs are used. The risk of food migraines by the consumption of fermented sausages is limited. The threshold of 6 mg TYR for patients, treated with the classical MAOI drugs (McCabe, 1986), is only exceeded when eating, for example, more than 68 g of the most heavily contaminated sausages. Products such as Dry sausage, Boerenring and Snacks (in exception of one outlier of 146.0 mg/kg) showed TYR values below 50 mg/kg. The small diameter of these sausages resulted in a low moisture content and a largely aerobic meat environment. Hence, the optimal conditions for the synthesis of decarboxylases were not met and therefore biogenic amine production and especially TYR levels remained low (Bover-Cid *et al.*, 1999a). Sausage samples with an increased TYR level often seems to show also the presence of low concentrations of PHE, probably attributed to the non-specific tyrosine decarboxylase activity of enterococci (Nakazawa *et al.*, 1977).

As described in the literature (Latorre-Moratella *et al.*, 2008), after TYR the most important biogenic amines in dry fermented sausages are PUT and CAD. In this study all samples were contaminated with PUT. Nevertheless, the CAD contamination rate of the samples collected on the Belgian market remained low. Due to the complex interaction between processing parameters, the quality of raw meat materials and the microbial flora in the product, a large variation in PUT and CAD concentrations is seen among the different analysed products. Boerenring has the lowest contamination rate for PUT and even no CAD was detected. A Gaumais sample, categorized as

Table 3.5 Biogenic amine contents in commercial dry fermented sausages, results presented as the median (min – max) concentration (mg/kg).

Type	n	TRYP[a,b]	PHE[a,b]	PUT[a,b]	CAD[a,b,]	HIS[a,b]	SER[a,b]	TYR[a,b]	SPM[a,b]	SPD[a,b]
North European type										
Boerenring (No. 1)	3	7.7 (nd-13.8)	nd (nd-6.5)	0.6 (0.5-1.2)	nd	nd	nd	7.1 (*1.9*-29.0)	9.1 (5.5-12.0)	3.3 (2.5-6.0)
Boulogne (No. 2)	3	45.8 (20.3-109.7)	3.4 (3.0-30.6)	97.5 (29.2-182.4)	3.9 (1.44-7.7)	nd	nd	140.7 (102.5-302.9)	5.5 (*2.5*-5.6)	2.0 (1.1-2.2)
Garlic salami (No. 6)	6	4.3 (nd-8.1)	1.4 (nd-5.2)	20.3 (0.4-143.2)	1.0 (nd-2.31)	nd	nd	59.8 (*1.3*-123.6)	6.5 (4.3-9.6)	3.2 (1.5-5.8)
German types (No. 7)	2	26.2 (5.3-47.2)	10.0 (nd-20.0)	73.5 (7.0-139.9)	27.7 (nd-55.4)	6.2 (nd-12.4)	nd	107.2 (15.7-198.6)	6.7 (4.3-9.2)	3.7 (1.2-6.2)
Light salami (No. 9)	5	6.5 (nd-29.4)	nd (nd-1.6)	3.8 (1.0-49.4)	0.4 (nd-2.5)	nd (nd-0.7)	nd	26.3 (22.0-113.9)	8.4 (4.6-9.1)	2.3 (1.7-6.1)
Pepper salami (No. 10)	4	3.6 (nd-7.2)	nd (nd-1.6)	8.4 (0.5-41.7)	0.3 (nd-1.9)	nd	nd	3.7 (*1.6*-85.4)	6.6 (4.6-10.6)	3.7 (0.8-5.1)
Salami (No. 11)	10	5.0 (nd-22.7)	0.9 (nd-10.8)	67.0 (0.8-229.0)	1.9 (0.3-246.8)	nd (nd-21.9)	nd	27.3 (*1.6*-174.1)	6.3 (nd-9.28)	1.6 (nd-3.7)
Smoked salami (No. 13)	10	5.3 (nd-32.2)	nd (nd-4.5)	1.5 (*0.3*-99.3)	0.4 (nd-2.2)	nd (nd-20.4)	nd	5.2 (nd-99.1)	8.4 (4.2-11.4)	5.0 (1.3-6.3)
Turkey salami (No. 15)	6	13.1 (5.4-56.2)	0.7 (nd-54.4)	4.2 (0.4-90.5)	1.1 (0.5-10.3)	nd (nd-131.0)	nd	19.1 (*1.6*-250.9)	5.6 (*2.8*-9.8)	4.1 (1.0-6.6)
South European type										
Chorizo (No. 3)	10	11.4 (*1.4*-105.4)	4.3 (1.3-38.1)	27.6 (*0.3*-183.6)	1.3 (*0.2*-380.1)	nd (nd-32.86)	nd	78.8 (*1.9*-242.7)	7.4 (3.5-21.1)	2.2 (nd-5.8)
Dry sausage (No. 4)	6	5.6 (4.4-7.0)	nd (nd-2.9)	6.7 (0.6-62.2)	3.5 (0.7-6.1)	nd	nd	10.0 (*1.2*-25.1)	6.1 (*2.6*-8.4)	*0.3* (nd-2.3)
French types (No. 5)	6	11.7 (4.0-92.4)	4.2 (1.6-21.0)	114.6 (0.7-316.4)	18.9 (1.6-641.4)	*2.3* (nd-54.1)	nd	113.4 (46.2-410.8)	5.3 (3.5-6.7)	1.2 (*0.5*-5.2)
Italian types (No. 8)	9	47.0 (3.4-99.8)	24.9 (2.2-57.1)	170.4 (1.9-236.8)	7.8 (0.3-68.7)	*1.4* (nd-65.9)	nd	149.9 (126.2-201.2)	5.4 (*2.3*-10.3)	0.7 (nd-6.6)
Saucisson d'Ardennes (No. 12)	6	7.3 (*2.6*-54.0)	1.1 (nd-42.8)	57.1 (*0.3*-182.0)	0.8 (nd-2.9)	nd (nd-80.6)	nd	68.0 (nd-210.7)	5.3 (3.6-9.6)	3.5 (*0.3*-7.4)
Snack (No. 14)	15	4.6 (nd-24.4)	nd (nd-26.3)	7.1 (0.5-124.1)	0.3 (nd-31.2)	nd (nd-51.1)	nd (nd-10.1)	3.6 (nd-146.0)	4.5 (nd-14.9)	2.1 (nd-13.3)
Total	101	6.2 (nd-109.7)	1.3 (nd-57.1)	9.3 (*0.3* – 316.4)	1.1 (nd – 641.4)	nd (nd-131.0)	nd (nd-10.1)	26.3 (nd-410.8)	6.1 (nd – 21.1)	2.3 (nd – 13.3)

[a]nd = not detected, values below the MDL, [b]values estimated below the MQL are printed in italics.

French type, showed the highest levels for PUT (316 mg/kg) and CAD (614 mg/kg). The high total biogenic amine content, (1234 mg/kg) was in big contrast to the other samples (median = 57 mg/kg). Of all investigated products, this is the only sample that - at the end of shelf life - exceeded the level of 1000 mg/kg, which has been suggested to be dangerous for human health (Silla Santos, 1996).

The concentrations of the SPM and SPD were limited with maximum levels of 21 mg/kg and 13 mg/kg, respectively. The small variability is explained by the natural origin of SPD and SPM in the meat. Almost no microbial synthesis takes place during the fermentation. During the storage, a small decline in SPD and SPM can occur, most likely due to deamination by microbial PAO enzymes (Razin *et al.*, 1959). This explains that at the end of shelf life the values are slightly lower than in other studies where measurements were performed earlier (Hernández-Jover *et al.*, 1997; Komprda *et al.*, 2004; Ruiz-Capillas & Jimenez-Colmenero, 2005).

3.3.4 OCCURRENCE OF VOLATILE *N*-NITROSAMINES

In 54 of the 101 samples, collected on the Belgian market, no volatile *N*-nitrosamines were detected at the end of shelf life. With 50% of the sausages being contaminated with *N*-nitrosamines, the South-European products scored just slightly higher than the North-European (43%). When *N*-nitrosamines were detected, their total amount remained below 5.5 µg/kg, except for one sample, which showed an exceptionally high total *N*-nitrosamine concentration of 14.0 µg/kg. This particular sample, categorized as a Pepper salami, was mainly contaminated by NPIP (12.3 µg/kg). Moreover, NPIP is also the most dominant *N*-nitrosamine in this study. NPIP is present above MDL (MDL = 0.8 µg/kg) in 28% of all sausages samples, but only 3% of the investigated sausages contained a concentration higher than the MQL-value of 2.5 µg/kg. As described in Chapter 1.3.3.2, biogenic amines, especially CAD and SPD are considered to be precursors for the NPIP. On the other hand, the use of piperine-containing spices such as black pepper, can supply the PIP ring necessary for the formation of NPIP.

With a contamination rate of 22% (above MDL of 0.6 µg/kg), NMOR is the second most common *N*-nitrosamine found in the dry fermented sausages. Except for one particular Chorizo sample (1.6 µg/kg), the detected NMOR concentrations remained below the MQL-value of 1.5 µg/kg. In contrast to NPIP, no relationship between the investigated biogenic amines and NMOR is expected, since the direct precursor is morpholine. It can be questioned how this presumed precursor could contaminate the dry fermented sausages, since the presence of morpholine is mainly due to the use of waxes in packaging materials or the use of anticorrosion agents in the meat factories (Domanska & Kowalski, 2003).

To a lesser extent (12%), the samples could also contain detectable amounts of NDMA (above MDL of 0.2 µg/kg). Two samples – a Turkey sample and a Light product – contained NDMA levels above the MQL value of 0.8 µg/kg.

The occurrence of NPYR was more rare in this study and only three samples – a Chorizo sample (0.8 µg/kg), a sausage with Garlic (0.3 µg/kg) and a Pepper salami (1.5 µg/kg) – were found to be contaminated with trace amounts of NPYR (between MDL of 0.5 µg/kg and MQL of 1.6 µg/kg). The presence of NPYR in the Chorizo sample can be explained by the use of a large quantity of paprika, which is known to contain pyrrolidine (Huxel *et al.*, 1974). The rare occurrence of NPYR in the dry fermented sausage samples can be explained by the lack of a heating step, needed for the efficient formation of NPYR from its precursors, like pyrrolidine (derived from PUT), proline and SPD (Drabik-Markiewicz *et al.*, 2010, 2011).

In none of the dry fermented sausage samples NDEA was detected (all below MDL of 0.3 µg/kg), while only two samples, classified as Saucisson d'Ardennes and Chorizo, showed NDBA contamination above MDL of 0.6 µg/kg. The concentrations remained below the MQL value of 2.0 µg/kg. Since the presence of NDEA and NDBA in meat products is related to the contact with rubber nettings and plastics (Sen *et al.*, 1993), it is obvious that the low contamination rate on the Belgian market proves the suitable quality of the used packaging materials.

3.3.5 EXPLORATORY DATA ANALYSIS

To obtain a better understanding of the relationships of the physical and chemical factors and the chemical composition of the samples in the different product categories, PCA and HCA were performed. For the data treatment, only the variables were considered which contained a considerable percentage of detectable values. As a consequence, the following 18 variables were considered: pH (AN_pH), a_w (AN_AW), salt (AN_SALT), moisture (AN_WATER), free fat (AN_FAT), protein (AN_PROT), moisture-protein ratio (CAL_MPR), residual sodium nitrite (AN_NO_2), residual sodium nitrate (AN_NO_3), TRYP, PHE, PUT, CAD, TYR, SPD, SPM, total biogenic amine (TOT_BA) and the total *N*-nitrosamine (TOT_NA) content. The individual *N*-nitrosamines were disregarded since the percentages of detectable values were too small.

In a first instance PCA was performed to reveal patterns among the different product categories. Three principal components (PC) were extracted which explained 63.6% (PC1: 29.4%, PC2: 25.8% and PC3: 8.4%) of the total variance. On the PC1-PC2 score plot (Figure 3.1) a distinction of regional origin of the sausages can be made along the PC1. The large majority of the North-European types (product categories No. 1, 2, 6, 7, 9, 10, 11, 13 and 15) are located on the positive part of the PC1-axis, while the South-European types (categories No. 3, 4, 5, 8, 12, and 14) are mainly situated on the negative part.

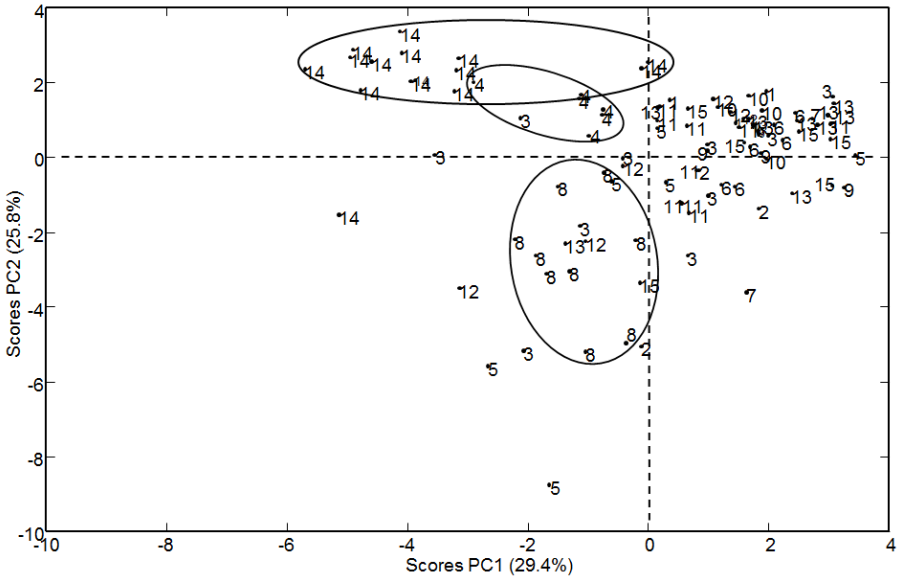


Figure 3.1 Score plot of the first two principal components, samples labeled according to the product category No.: Boerenring (1), Boulogne (2), Chorizo (3), Dry sausage (4), French type (5), Garlic salami (6), German type (7), Italian type (8), Light salami (9), Pepper salami (10), Salami (11), Saucisson d'Ardennes (12), Smoked salami (13), Snack (14) and Turkey salami (15).

Based on the PC1-PC2 loading plot (Figure 3.2), the differentiation seemed, in a first instance, to be attributed to the SPD, PHE and PUT concentrations. In the boxplot of PHE (Figure 3.3, A), the Southern products have higher PHE concentrations, while the Northern products scarcely contain PHE. However, for the SPD contents, no clear differences between North and South-Europe could be observed in the boxplot (Figure 3.3, B). For PUT (Figure 3.3, C), the median concentrations of both groups are low but in the Southern group greater numbers of high concentrations are seen.

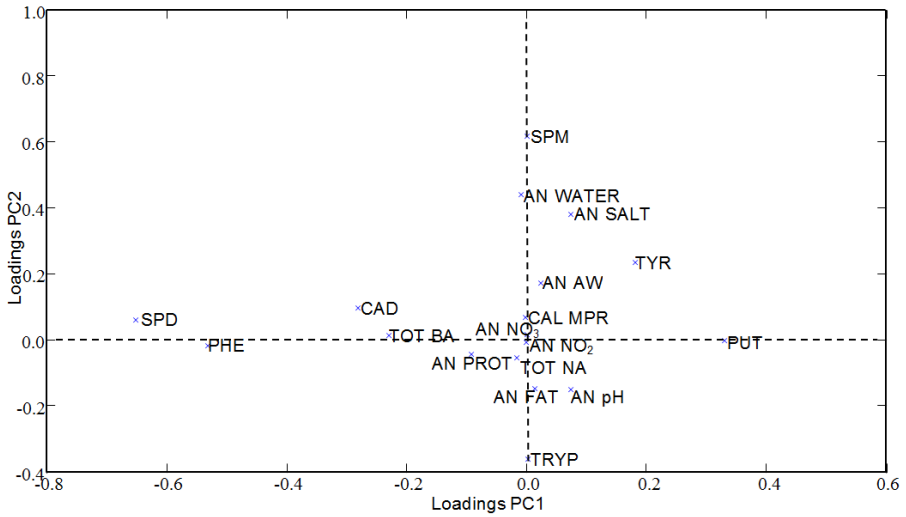


Figure 3.2 Loading plot of the first two principal components, variables: pH (AN_pH), a_w (AN_AW), salt (AN_SALT), moisture (AN_WATER), free fat (AN_FAT), protein (AN_PROT), moisture-protein ratio (CAL_MPR), residual sodium nitrite (AN_NO_2), residual sodium nitrate (AN_NO_3), TRYP, PHE, PUT, CAD, TYR, SPD, SPM, total biogenic amine (TOT_BA) and the total *N*-nitrosamine (TOT_NA) content.

Considering the Southern products, the Snack sausages (No. 14) are mainly clustered in the upper left quadrant of Figure 3.1 (negative PC1, positive PC2), while the Italian Style sausages (No. 8) are situated at a negative PC2. The products of the category Dry sausages (No. 4) are located in between, but have also positive PC2 scores. The location of these three groups on the PC2-axis is mainly dominated by the concentrations of SPM (positive PC2 loading) and to a lesser extent of TRYP (negative PC2 loading) and of the variables AN_WATER and AN_SALT (Figure 3.2). However, when considering the categories No. 4 and 14 (Dry sausage and Snacks) versus category No. 8 (Italian types), SPM does not show a clear difference (Table 3.5), while the three other variables can be considered different. The TRYP concentration (Table 3.5) and AN_WATER (moisture content; Table 3.3) are clearly lower in the categories No. 4 and 14 than in the category No. 8. For AN_SALT the opposite is seen (NaCl; Table 3.3).

A distinction between the different Northern classes (right side in Figure 3.1) could not be made. This indicates that only limited intrinsic differences in the physical and chemical properties and the biogenic amine contents among the Northern product categories could be observed.

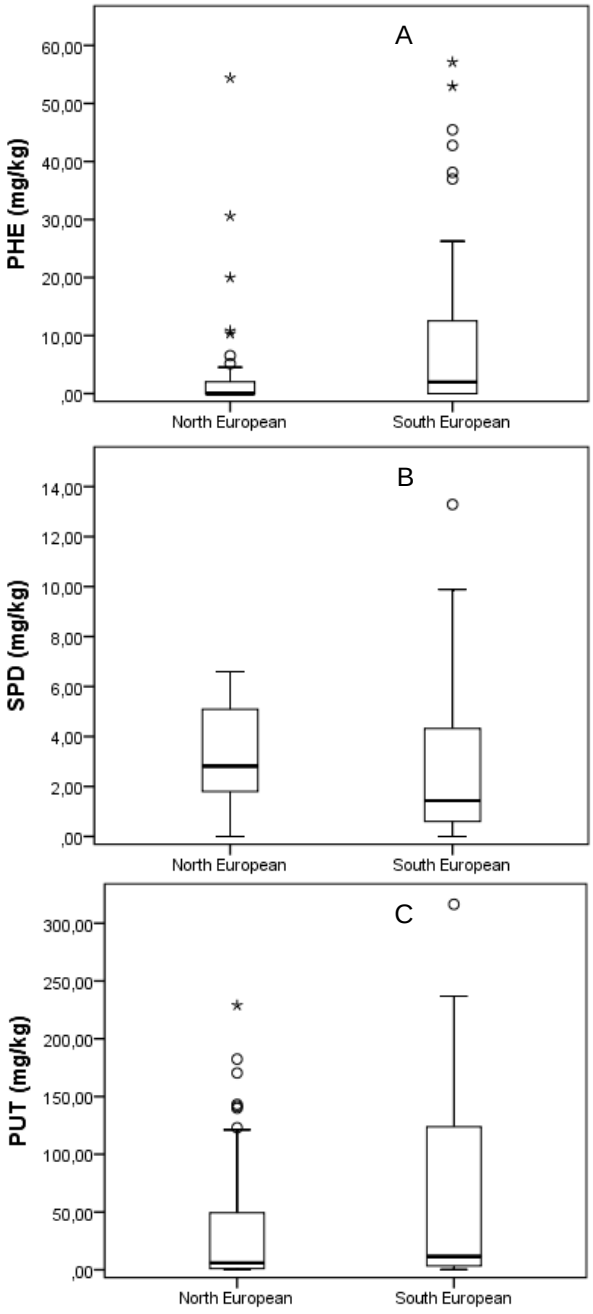


Figure 3.3 Boxplots of PHE (A), SPD (B) and PUT (C) for each regional origin.

In summary, the principal component analysis was able to discriminate some of the product categories. However, this clustering was mainly attributed to variance of some specific biogenic amine concentrations. None of the product categories could be related to an increased *N*-nitrosamine risk since the variable TOT_NA was situated in the center of the loading plot (low PC1 and PC2 loading).

The HCA of the samples confirmed the observations seen in the PCA plots but did not provide additional information (dendrograms not shown).

A HCA of the variables is given in the dendrogram in Figure 3.4. All the biogenic amines, except the natural polyamines SPD and SPM can be found in one cluster. The variables PUT and TYR showed most similarity with the total biogenic amine content. In accordance to many other studies concerning the biogenic amines content in dry sausages (Suzzi & Gardini, 2003; Komprda *et al.*, 2004), it can be concluded that PUT and TYR are dominating the biogenic amine profile of dry fermented sausages.

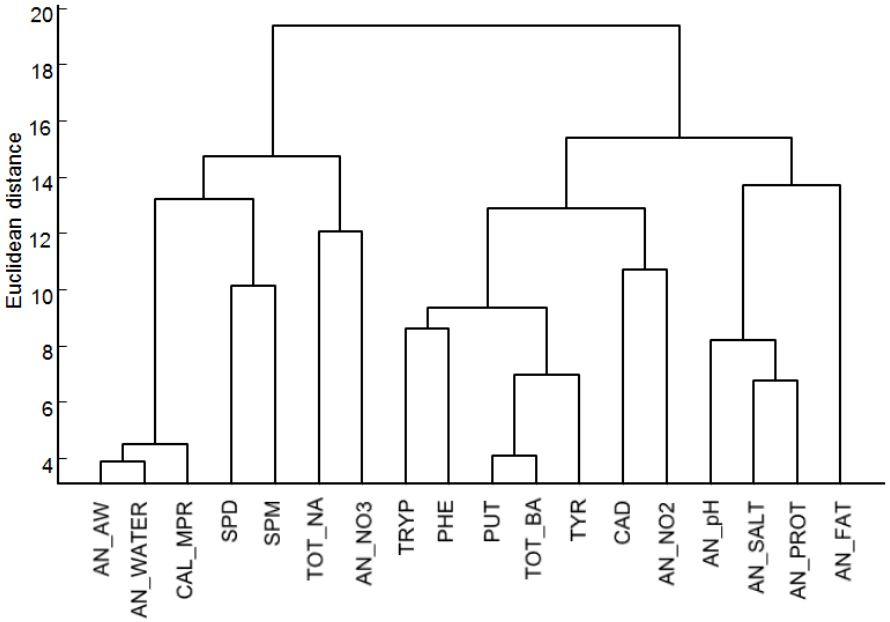


Figure 3.4 Dendrogram for the variables, using Euclidean distances as similarity measure with complete linkage to merge clusters.

The natural polyamines (SPD and SPM) are clustered to a lesser extent with the physical and chemical variables related to the water content of the product (AN_AW, AN_WATER and CAL_MPR). This relationship can be explained by the fact that SPM and SPD are decreasing during the ripening (Parente *et al.*, 2001). As a consequence, lower amounts of these

polyamines will be related to an increased extent of drying which occurs during an extended ripening period.

For the formation of *N*-nitrosamines, the availability of secondary amines and a nitrosating agent is required (Honikel, 2008; Drabik-Markiewicz *et al.*, 2011). From the latter variables, investigated in this study, only the residual $NaNO_3$ concentration could be linked to a given extent to the total *N*-nitrosamine content (Figure 3.4). No clustering is seen with the investigated biogenic amines.

3.4 CONCLUSIONS

The biogenic amine accumulation in dry fermented sausages at the end of shelf life remains low. Only in one sample the total biogenic amine content reached critical levels (1000 mg/kg), mainly caused by the accumulation of PUT, CAD and TYR. Although it is assumed that biogenic amines, such as CAD, PUT and the natural polyamines SPD and SPM, are potential precursors of *N*-nitrosamines, no relationships were observed.

In this study, only a limited relation of the *N*-nitrosamine content was observed with the residual $NaNO_3$ level and no relationship with $NaNO_2$ level. It can be assumed that the amounts of carcinogenic *N*-nitrosamines remained low because the median concentrations of residual $NaNO_2$ and $NaNO_3$ levels were lower than 20 mg/kg in the screened products.

Only NMOR and NPIP were detected in a relatively high number of samples (22% and 28%, respectively), but quantifiable levels were rarely measured. The incidence of *N*-nitrosamine contamination could not be linked to specific product categories or physical and chemical variables.

Given the higher prevalence of NPIP and the absent link with tested biogenic amines, it would be interesting to study in detail the NPIP formation in dry fermented sausages. For instance, it can be assumed that the NPIP formation may be attributed to the presence of PIP, originating from the use of black pepper. In addition the influence of additives (sodium nitrite and ascorbate) and process conditions (pH and a_w) on the NPIP formation will be studied.

CHAPTER 4
DEVELOPMENT OF A DRY FERMENTED SAUSAGE MODEL

4.1 INTRODUCTION

The production of dry fermented sausages is a traditional way of conserving meat. Although nowadays a strong industrialization characterizes the meat production, a wide variety of recipes are still in use and are often linked to regional traditions. Specific flavour profiles are generated by the use of dominating spices or particular ingredients. Besides the fermentation and drying, the microbial stability of the product can be enhanced by the use of additives like nitrite, and/or the application of smoke or fungal surface inoculation (Toldrá, 2007).

In a number of studies the presence of several *N*-nitrosamines in dry fermented meat products is mentioned (Ellen *et al.*, 1986; Mavelle *et al.*, 1991; Yurchenko & Mölder, 2007; Ozel *et al.*, 2010). To elucidate the *N*-nitrosamine formation in dry fermented sausages, the development of a representative sausage model is required. In Chapter 3 it was demonstrated that no sausage type more than another could clearly be identified to show a higher prevalence of *N*-nitrosamines contamination. Therefore, the sausage model will be based on the production of a general North European style dry fermented sausage preparation. To ensure a reliable sausage model, the production, more specifically the fermentation and drying, was monitored. Therefore the pH and weight loss, microbial counts, nitrite and nitrate content, were evaluated during the production. In addition, the quality of the sausages was characterized by determining the physical and chemical properties of the end product.

4.2 MATERIAL AND METHODS

4.2.1 EQUIPMENT AND MATERIALS

For the production of the dry fermented sausages, following equipment is used: a guillotine cutter (Magurit Gefrierschneider, Remscheid, Germany), a meat mincer (Minerva Omega Group, Bologna, Italy), a vacuum bowl cutter (Kilia, Neumünster, Germany), a sausage stuffing machine (Industrial Fuerpla, Valencia, Spain), a climate chamber (Kerres Anlagensysteme GmbH, Backnang, Germany), a pH-meter (Knick Portamess, Elscolab, Terschuur, The Netherlands), a vacuum packaging system (Webomatic Maschinenfabrik, Bochum, Germany), and a homogenizer (Foss Homogenizer 2096, Höganäs, Sweden). For stuffing the sausages, collagen casings with a diameter of 90 cm were purchased at Naturin (Weinheim, Germany).

4.2.2 INGREDIENTS OF THE SAUSAGE PRODUCTS

Fresh deboned and defatted shoulder meat (pork, *m. Triceps brachii*) and frozen (-16 °C) porcine back fat was purchased from a local wholesale meat supplier. The meat was divided into two portions, whereof one part was

frozen to -12 °C and the second part was kept refrigerated (4 °C). The starter culture, a mixture of *Lactobacillus sakei*, *Staphylococcus carnosus* and *S. xylosus* was purchased as Texel SA-306 (Danisco, Dangé-Saint-Romain, France). Sodium chloride, sodium ascorbate, dextrose, white pepper and nutmeg were provided by Rejo (Eke-Nazareth, Belgium) and sodium nitrite was from VWR International (West Chester, PA, USA).

4.2.3 SAUSAGE PRODUCTION

The production process of the dry fermented sausage model is illustrated in Figure 4.1 and schematically presented Figure 4.2. To prepare the raw materials for the sausage production, the frozen meat and fat portions were separately chopped into the guillotine cutter to obtain cubes of ca. five by ten cm (Figure 4.1 A). The refrigerated fresh meat portion was ground through a 4 mm plate (Figure 4.1 B). The sausage batter was prepared by cutting the frozen meat (3.75 kg) into the bowl cutter until particles of ca. 5 mm were achieved (Figure 4.1 C), where after the starter culture was divided over the meat. Subsequently, the backfat (2.68 kg) was added and was cutted. The obtained meat-fat mixture was seasoned by adding the additives: dextrose (7 g/kg), sodium ascorbate (0.5 g/kg), white pepper (2 g/kg) and nutmeg (0.4 g/kg). Finally, the binding was achieved by mixing the fresh meat fraction (3.21 kg), salt (28g NaCl/kg) and sodium nitrite (150 mg/kg) into the meat batter.

After the cutting and mixing process, the meat batter was stuffed in collagen casings (Figure 4.1 D) and hung on a trolley (Figure 4.1 E). The production time consisted of a fermentation and ripening stage. The sausages were fermented for three days at a temperature of 24 °C and a relative humidity (RH) of 94%. The fermentation process was monitored by measuring the pH of the sausages (Figure 4.1 F). During this fermentation stage, the sausages were smoked. Subsequently, a ripening stage (14 °C/85% RH) took place with the intention to dry the sausages until a weight loss of 20% or more was achieved.

4.2.4 SAMPLING

In order to evaluate the production process (pH and weight loss, microbial counts and nitrite and nitrate contents), the dry fermented sausages (n = 3) were sampled after the meat batter preparation (day 0), after the fermentation (day 3), during the ripening (day 7 and 14), and at the end of production (day 21). The samples collected at this last sampling point (day 21) were also used to determine the physical properties and chemical composition of the end product. For the microbial counts, the samples were immediately analysed. For the other analyses, the sausages were homogenized, vacuum packed and stored at -18 °C until analysis.

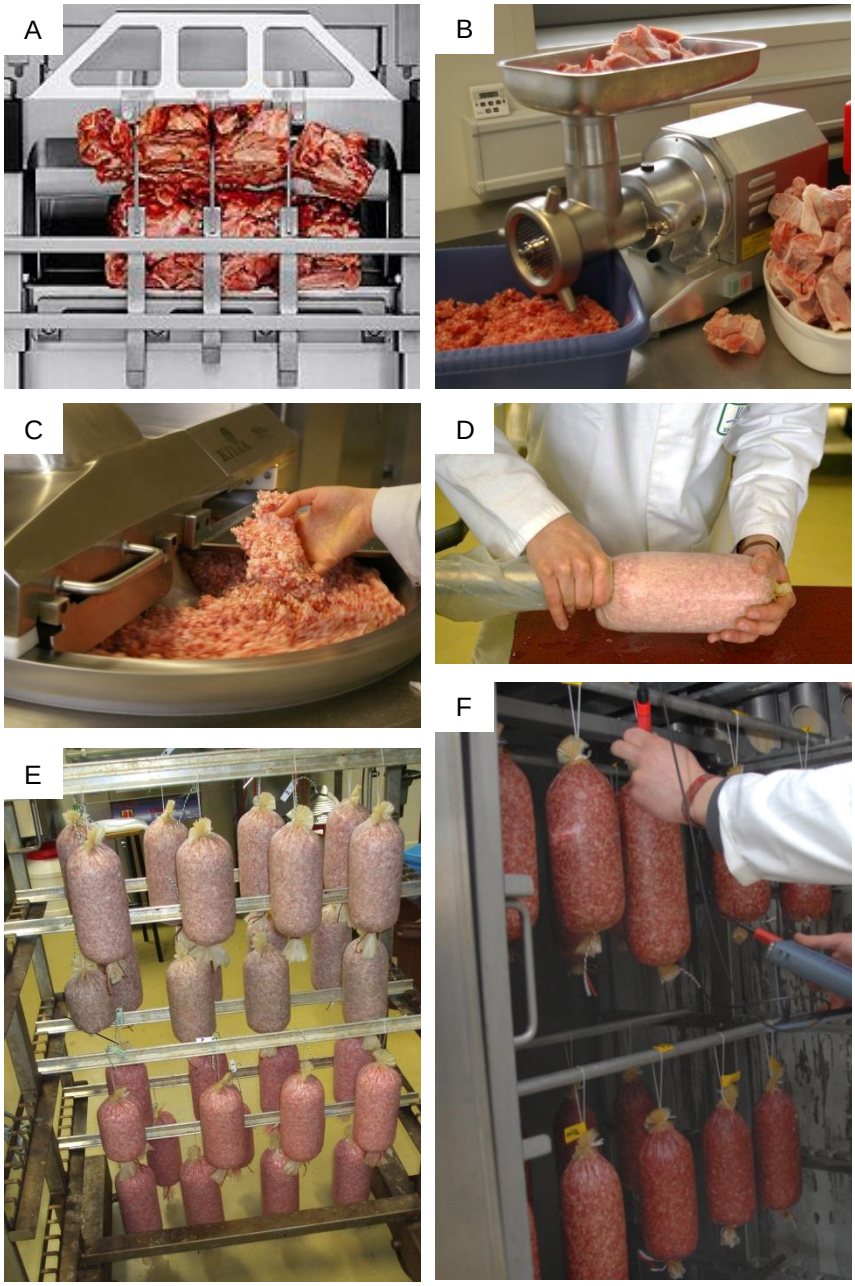


Figure 4.1 Stages in the production of dry fermented sausages: (A) chopping the frozen meat, (B) grinding the fresh meat, (C) cutting and mixing the meat and fat, (D) stuffing in collagen casings, (E) rest period before fermentation, (F) monitoring the pH during fermentation.

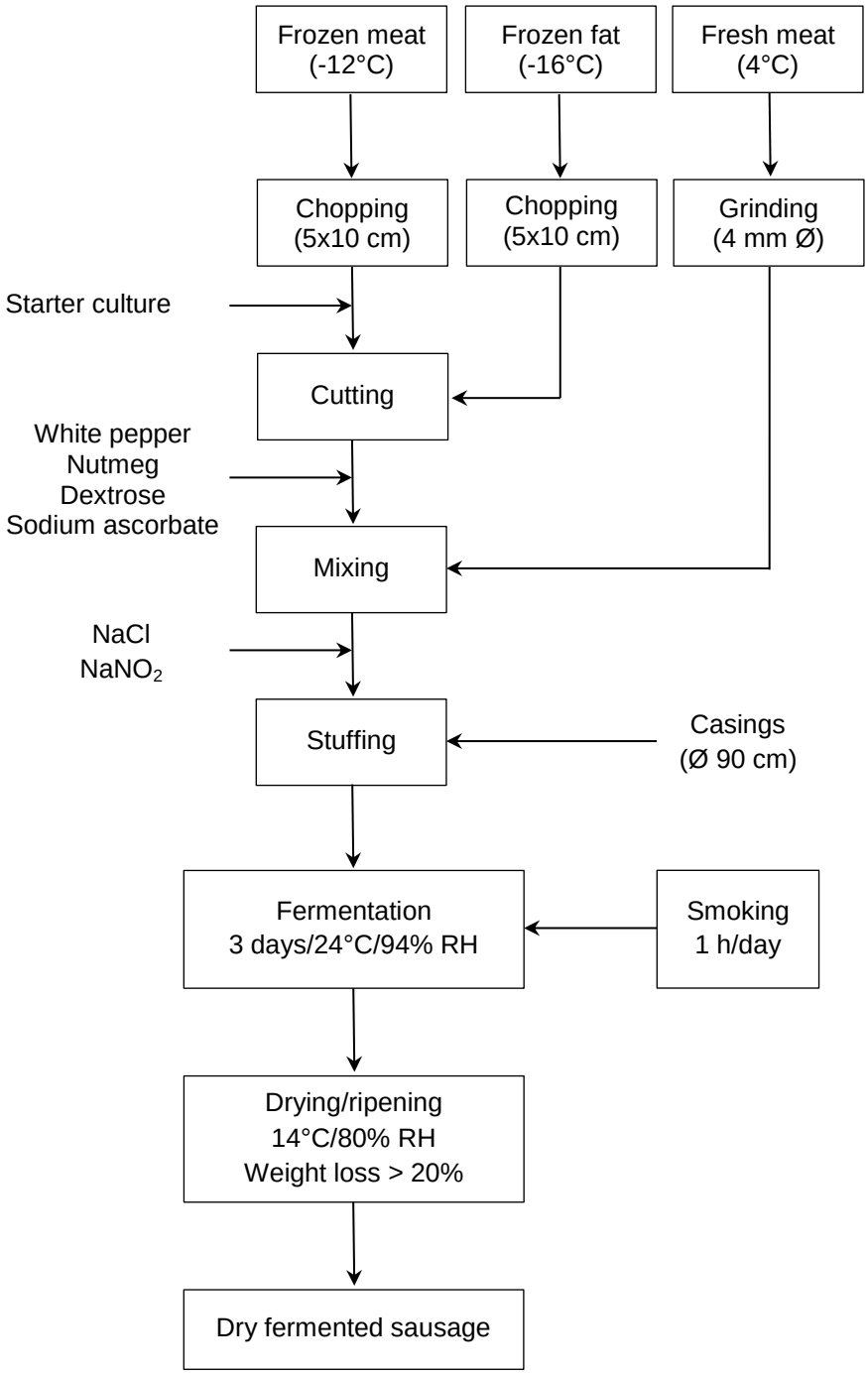


Figure 4.2 Flow diagram for the processing of dry fermented sausages.

4.2.5 PHYSICAL AND CHEMICAL ANALYSES

The physical analyses (pH and a_w), and the chemical analyses (moisture, protein, free fat and salt content) are described in Chapter 3.2.2.

The residual $NaNO_2$ and $NaNO_3$ levels were measured according the method described in Chapter 3.2.3.1. In this chapter, the concentrations were expressed on dry matter (DM). The MDL and MQL were 2 mg/kg DM and 10 mg/kg DM, respectively.

4.2.6 MICROBIAL COUNTS

The samples were aseptically homogenized with a stomacher (Masticator Classic 400, IUL Instruments, Barcelona, Spain). Decimal dilution series were prepared with sterile ringer solution (Oxoid, Basingstoke, England) and plated with the spiral plater (Eddy Jet, IUL Instruments). The following analyses were done: (1) LAB on de Man, Rogosa and Sharpe agar (MRS, Merck, Darmstadt, Germany) incubated witLLh a double layer at 30 °C for 72 h, (2) gram-positive coagulase positive cocci (GCC+) on mannitol salt agar (MSA, Merck) incubated at 30 °C for 48h, (3) *Enterobacteriaceae* on violet red bile glucose agar (VRBG, Biokar, Beauvais, France) incubated with a double layer at 30 °C for 48 h.

4.3 RESULTS AND DISCUSSION

4.3.1 EVALUATION OF THE PRODUCTION PROCESS

A successful dry fermented sausage production depends on the optimum growth of the starter culture during the fermentation, followed by a controlled drying of the product. For this, the sausages were fermented and ripened in a climate chamber, where the environmental temperature and relative humidity can be manipulated and monitored (Figure 4.3 A).

For the production of North European style sausages, the temperature and relative humidity were set at 24 °C and 94% RH during the three-days fermentation period. In order to dry the sausages thereafter, the temperature and RH were lowered to 14 °C and 80% RH, respectively. These conditions were maintained between the third and 21st day of production, resulting in a weight loss of 25%

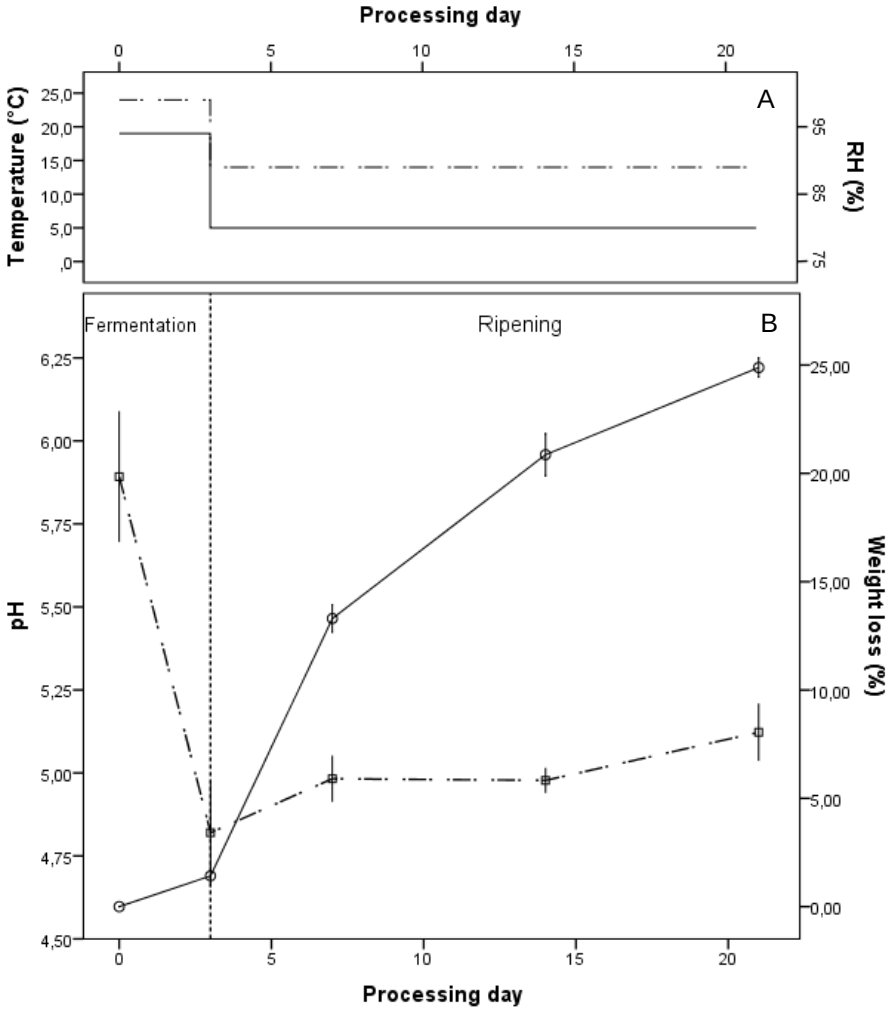


Figure 4.3 Processing conditions of the climate chamber (A); the temperature (-— -) and RH (—), and the changes in the product characteristics (B); the pH (-□-) and percentage weight loss (-o-), during the fermentation and ripening stages of the dry fermented sausage model.

Together with the optimal environmental conditions, the addition of dextrose resulted in an explosive growth of the starter culture during the fermentation period. As can be seen in Figure 4.4, the LAB population can reach a maximum count (above 9 log cfu/g) during the three-days fermentation phase. This microbial population metabolised the dextrose to lactic acid. As a consequence, the addition of 7g/kg dextrose resulted in a pH decrease of 1 unit (from ca. 5.9 to 4.9, see Figure 4.3 B). However, during the consecutive ripening period, the pH of the sausage slightly increased again due to the proteolytic activity of bacteria and remaining endoprotease activity of the muscle enzymes (Bolumar *et al.*, 2001).

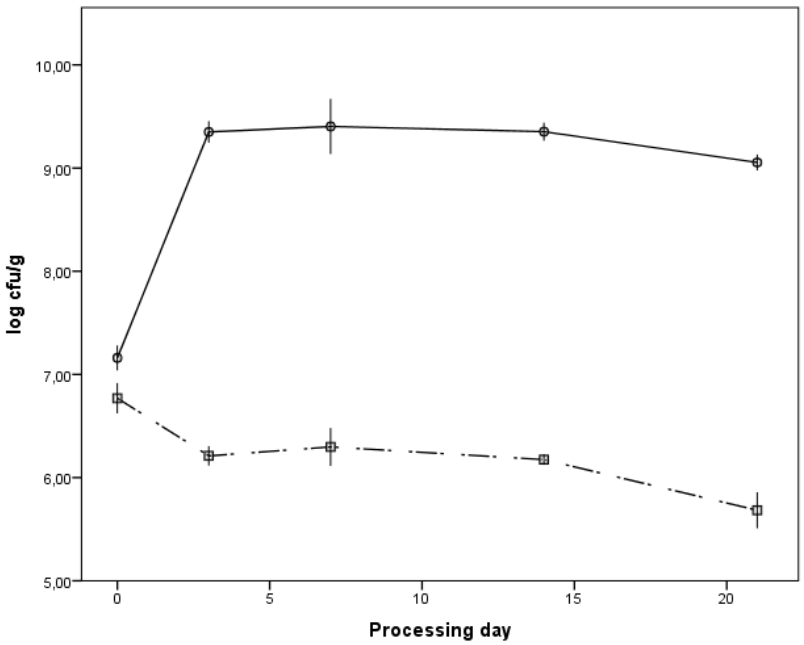


Figure 4.4 LAB (-o-) and GCC+ (-□-) counts during the fermentation and ripening stages of the dry fermented sausages.

Besides the LAB (*L. sakei*), the starter culture contained GCC+ bacteria, namely *S. xylosus* and *S. carnosus*. On the one hand, these staphylococci can reduce nitrate to nitrite in order to enhance the colour formation. On the other hand, they improve the flavour by proteolytic and lipolytic activity (Ravyts *et al.*, 2010). As can be seen in Figure 4.4, the initially added GCC+ population contained ca. 7 log cfu/g. However, the population decreased 1 log cfu/g during the fermentation and the consecutive ripening period. This reduction is mainly attributed to the poor competition capacity of the GCC+ against the presence and growth of the LAB population (Toldrá, 2007).

Although the GCC+ population of the starter is able to reduce nitrate, it was chosen not to add nitrate. Instead, sodium nitrite was directly added. In this way, the time consuming resting phase, necessary to activate the nitrate-reducing starter, was avoided. The legally permitted amount of sodium nitrite, namely 150 mg/kg (or ca. 350 mg/kg DM) was added to enhance the colour formation. Moreover, the addition of nitrite inhibits the growth of gram negative bacteria such as *Enterobacteriaceae* (Sanz *et al.*, 1997). As a result, the microbial counts on the VRBG plates remained below the detection limit of 2 log cfu/g during the whole production process.

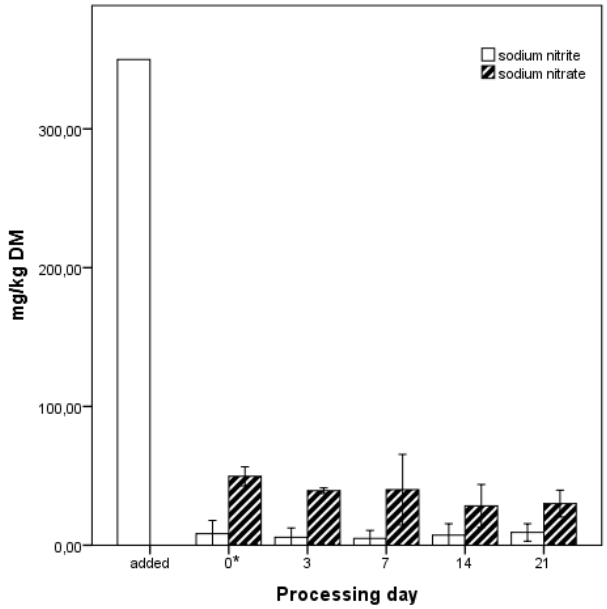


Figure 4.5 Sodium nitrite and nitrate concentrations during the production process of the dry fermented sausage model (*day 0: directly after stuffing).

As can be seen in Figure 4.5, the added nitrite was degraded very fast in the dry fermented sausage model. Directly after stuffing (day 0) only 2.4% of the initial added amount of sodium nitrite could be detected. The nitrite degradation is mainly attributed to the conversion to nitric oxide (NO) and a subsequent binding to myoglobin. As a consequence, the pigment nitrosomyoglobin will give the cured meat its typical red colour (Honikel, 2008). Although no nitrate was added, residual sodium nitrate levels were measured during the production of the dry fermented sausage model. Similar results were obtained in other studies (Cassens *et al.*, 1979). The occurrence of nitrate in nitrite cured meat products can be explained by the conversion of nitrite to the unstable anhydride (N_2O_3) (see Chapter 1.3.1), which on its turn is in equilibrum with NO and NO_2. The oxide NO_2 may react with water to form again one molecule of nitrous acid and one molecule nitric acid. Consequently residual amounts of both salts (nitrite and nitrate) can be present in the meat batter (Honikel, 2008).

4.3.2 CHARACTERIZATION OF THE END PRODUCT

The physical and chemical composition of the end product is given in Table 4.1. After a production period of 21 days the sausages had lost more than 25% of weight, resulting in a reduced a_w value, comparable to the commercial North European sausages investigated in Chapter 3. The moisture-protein ratio (MPR) of the dry fermented sausage model is 2.3:1, which is somewhat higher than the average MPR (2.2:1) of the commercial North European sausages (Chapter 3), mainly attributed to the slightly higher fat content of the model.

Table 4.1 Physical and chemical characteristics of the dry fermented sausage model at the end of production.

Physical analysis		Chemical analysis (g/100g)	
a_w	0.93 ± 0.01	moisture	42.9 ± 2.5
pH	5.1 ± 0.1	protein	19.0 ± 2.2
		free fat	32.0 ± 1.7
		NaCl	3.5 ± 0.1

Furthermore, the microbial stability of the dry fermented sausage model can be achieved by the use of nitrite, a lowered pH and reduced water activity (Leistner, 1995). In conclusion, the sausage model can be categorized as a shelf-stable fermented meat product since it meets the European requirements (pH < 5.2 and a_w < 0.95) (Ockerman & Basu, 2007).

4.4 CONCLUSIONS

A dry fermented sausage model was produced under controlled fermentation and drying conditions. Hereby the LAB, added as a part of the starter culture, could acidify the product to a pH of 4.9. Simultaneously, the added amount of nitrite was degraded to trace levels in order to bind as nitrogen oxide to myoglobin. Subsequently the product was dried until a weight loss of 25%. After 21 days the end product was achieved, characterized by a final pH of 5.1 and an a_w of 0.93. Meeting the requirements of a shelf-stable fermented meat product, it can be concluded that a representative dry fermented sausage model was prepared.

CHAPTER 5
EVALUATION OF THE *N*-NITROSOPIPERIDINE FORMATION IN DRY FERMENTED SAUSAGES[4]

[4] This chapter was based on following paper:
De Mey, E., De Maere, H., Goemaere, O., Steen, L., Peeters, M.-C., Derdelinckx, G., Paelinck, H., Fraeye, I. (2013). Evaluation of *N*-nitrosopiperidine formation from biogenic amines during the production of dry fermented sausages, Food and Bioprocess Technology, doi: 10.1007/s11947-013-1125-5.

5.1 INTRODUCTION

In Chapter 3, it was described that commercial dry fermented sausages contain especially NPIP. In the literature, the biogenic amine CAD is considered as a potential precursor of NPIP. However, this primary amine must firstly be transformed to the alkaloid PIP (Shalaby, 1996). In a heated lean meat model, the addition of CAD and nitrite concentrations, higher than legally permitted, induced NPIP formation. However, only in the case that very high processing temperatures were used (Drabik-Markiewicz *et al.*, 2011). Moreover, the aforementioned *N*-nitrosamine was formed much more easily when PIP was directly added to the model (Drabik-Markiewicz *et al.*, 2010). Concerning the presence of potential precursors, dry fermented sausages can on the one hand be a source of relative high amounts of biogenic amines. On the other hand, sausages can contain PIP since this alkaloid is a degradation product of piperine, which is the main pungent compound of pepper (González-Mancebo *et al.*, 2004).

The contribution of CAD and PIP is demonstrated in heated cured meat products. However, the NPIP formation in dry fermented sausage must be induced by other parameters as there is no heating step during the production. Meat products in general have a pH value between 5.5 and 6.2, while dry fermented sausages can have a pH in the range of 4.5 - 5.5 (Toldrá, 2007). It can be assumed that the *N*-nitrosamine formation can occur more easily in dry fermented sausages since the pH of the product comes closer to the pH optimum (pH 3.5) of the nitrosation reaction (Pegg & Shahidi, 2000).

The aim of the study was to estimate the risk of *N*-nitrosamine formation in relation to the biogenic amine accumulation during the production of dry fermented sausages. For that purpose the evolution of the biogenic amine content was assessed and the formation of NPIP was studied by artificial addition of CAD and PIP to a dry fermented sausage model. Hereby the influence of the pH and $NaNO_2$ addition on the biogenic amine accumulation and *N*-nitrosamine formation was investigated. Moreover, the influence of the availability of the nitrosating agent on the NPIP formation from PIP was studied.

5.2 MATERIAL AND METHODS

5.2.1 EXPERIMENTAL SET-UP

Different batches of dry fermented sausage were made according the sausage model described in Chapter 4. As can be seen in Table 5.1, variations in the sausage formulation were included. A first trial (I) comprised 12 preparations, whereby different NPIP precursors were added, i.e., none (A), CAD (B) or PIP (C). Furthermore preparations both with and without $NaNO_2$ were prepared, to evaluate the role of nitrite on the accumulation of

biogenic amines and the formation of *N*-nitrosamines. Finally the amount of dextrose was varied to evaluate the role of pH in the *N*-nitrosamine formation. Since dextrose can be converted to lactic acid by LAB, the high and low dose of dextrose induced respectively a strong and mild acidification during the fermentation.

Table 5.1 Overview of the variations in the formulation of the different preparations.

Trial	No.	Precursor	$NaNO_2$ (mg/kg)	Sodium ascorbate (mg/kg)	Dextrose (g/kg)
I	A1	-	0	500	3
	A2	-	0	500	7
	A3	-	150	500	3
	A4	-	150	500	7
	B1	500 mg/kg CAD.2HCl	0	500	3
	B2	500 mg/kg CAD.2HCl	0	500	7
	B3	500 mg/kg CAD.2HCl	150	500	3
	B4	500 mg/kg CAD.2HCl	150	500	7
	C1	10 mg/kg PIP	0	500	3
	C2	10 mg/kg PIP	0	500	7
	C3	10 mg/kg PIP	150	500	3
	C4	10 mg/kg PIP	150	500	7
II	C2	10 mg/kg PIP	0	500	7
	C4	10 mg/kg PIP	150	500	7
	C5	10 mg/kg PIP	150	0	7
	D2	100 mg/kg PIP	0	500	7
	D4	100 mg/kg PIP	150	500	7
	D5	100 mg/kg PIP	150	0	7

The second trial (II) comprised 6 preparations, whereby PIP concentrations were added, respectively 10 mg/kg (C) and 100 mg/kg (D). To evaluate the availability of nitrite as nitrosating agent during the production, the addition of sodium nitrite and sodium ascorbate was varied. Since sodium ascorbate can enhance the nitrite reduction, the degradation rate of $NaNO_2$ can be influenced by the variation of this commonly used reductans.

5.2.2 SAMPLING AND ANALYSES

Analogous to Chapter 4.2.4, dry fermented sausages (n = 3) of each preparation were sampled after the meat batter preparation (day 0), after the fermentation (day 3), during the ripening (day 7 and 14), and at the end of production (day 21). In this chapter the LAB count, pH, weight loss and residual nitrite were determined according the methods described earlier (Chapter 4.2.5 and 4.2.6). In addition, the biogenic amines and *N*-nitrosamines content was determined during the production of the sausages.

The biogenic amines content of the dry fermented sausage samples were determined by the HPLC method as described in Chapter 2. In this study, the concentrations were expressed in mg/kg of DM. The biogenic amines of interest were PIP (MDL = 0.3 mg/kg DM, MQL = 0.7 mg/kg DM), PUT (MDL = 0.3 mg/kg DM, MQL = 0.8 mg/kg DM), CAD (MDL = 0.2 mg/kg DM, MQL = 0.6 mg/kg DM), and TYR (MDL = 1.9 mg/kg DM, MQL = 6.4 mg/kg DM).

The *N*-nitrosamines were analysed according the method of Drabik-Markiewicz (2010) (Chapter 3.2.3.3). The following volatile *N*-nitrosamines could be separated and detected: NDMA (MDL = 0.4 µg/kg DM, MQL = 1.1 µg/kg DM), NDEA (MDL = 0.5 µg/kg DM, MQL = 1.6 µg/kg DM), NDBA (MDL = 1.1 µg/kg DM, MQL = 3.6 µg/kg DM), NPIP (MDL = 0.9 µg/kg DM, MQL = 2.7 µg/kg DM), NPYR (MDL = 0.9 µg/kg DM, MQL = 2.9 µg/kg DM) and NMOR (MDL = 0.9 µg/kg DM, MQL = 2.7 µg/kg DM).

5.2.3 STATISTICAL EVALUATION

Due to a large decrease in moisture content during the ripening process, the concentrations are calculated on a dry matter base and are shown in the tables and figures as the mean values ± standard deviation.

The data of the experiments were statistically analysed by PASW Statistics 19.0.0 (SPSS Inc.). A significance level of $\alpha < 0.05$ was employed for all tests. For the production monitoring (pH, LAB counts, weight loss and residual sodium nitrite concentration), one-way analyses of variance (ANOVAs) for each sampling day were performed. Afterwards, a planned contrasts analysis was introduced to compare the effect of the dextrose dose on the pH, LAB counts, and weight loss. The Duncan's post hoc comparison of means test was used to compare the influence of the addition of sodium nitrite and sodium ascorbate on the residual sodium nitrite level.

In the first trial, the effect of (x_1) production day (0, 3, 7, 14 and 21), (x_2) sodium nitrite (0 or 150 mg/kg) and (x_3) pH (5.3 or 4.9) on the accumulation of the individual biogenic amines were analysed by three-way ANOVA. The influence of production day (x_1), sodium nitrite (x_2), and pH (x_3) on the NPIP formation in the data set PIP (C) was analysed by a three-way ANOVA.

In the second trial, a three-way ANOVA was performed to evaluate the NPIP formation, using the factors (x_1) production day, (x_2) PIP concentration (10 or 100 mg/kg), and (x_3) nitrosating agent (0 mg/kg $NaNO_2$ + 500 mg/kg sodium ascorbate, 150 mg/kg $NaNO_2$ + 500 mg/kg sodium ascorbate, 150 mg/kg $NaNO_2$ + 0 mg/kg sodium ascorbate).

5.3 RESULTS AND DISCUSSION

5.3.1 TRIAL I

5.3.1.1 PRODUCTION MONITORING

The fermentation and ripening of the dry fermented sausages were monitored to ensure a controlled production of all the preparations (see Chapter 4). In this way, any differences in the *N*-nitrosamine formation can be related to the addition of nitrite or precursors and are not influenced by accidental differences in the production process.

The addition of dextrose, as energy source for the starter culture, was varied in the first trial, with the purpose of producing sausages with different final pH values. Upon addition of 3 g dextrose per kg meat batter, the LAB population was able to grow to 10^8 cfu/g, while the addition of 7g/kg resulted in a significantly higher LAB count of 10^9 cfu/g (Figure 5.1). As a consequence, the addition of the low dextrose dose resulted in a moderate pH-drop from 6.1 to 5.3 by the end of the fermentation stage (day 3), while by the addition of the high dextrose dose, the sausages were significantly more acidified (pH 4.9) (Figure 5.2).

As can be seen in Figure 5.3, the weight losses increased rapidly once the ripening process was started (at day 3). At day 21, an average weight loss of 23.4 ± 0.9% was achieved and no significant difference in drying rate was observed.

Besides the variation in pH, the amount of sodium nitrite was also varied. Although half of the batches were prepared with the addition of 150 mg/kg $NaNO_2$, all the residual $NaNO_2$ concentrations were, directly after stuffing of the sausages, lower than the MQL-values. This is an indication of the fast degradation of nitrite in the presence of sodium ascorbate (Gøtterup, 2008), as also was observed in Chapter 4.3.1.

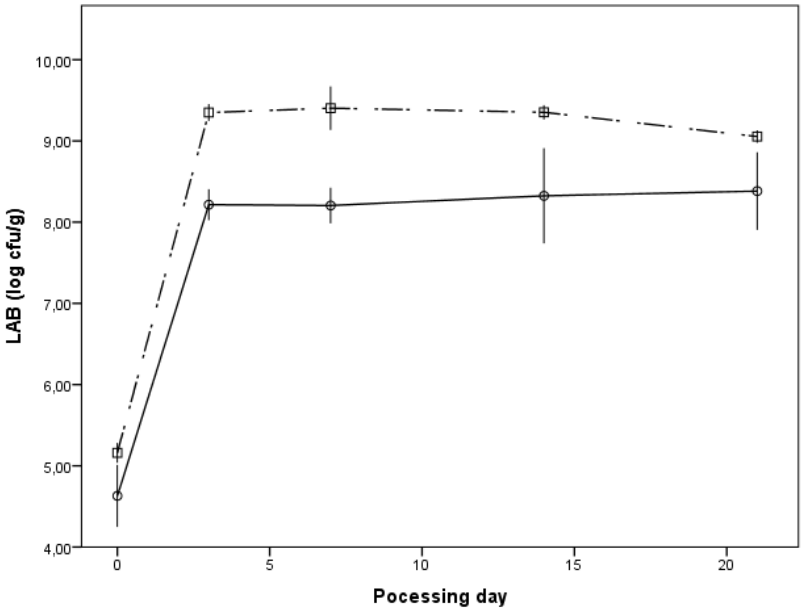


Figure 5.1 LAB counts during the fermentation and ripening stages of sausages prepared with 7 g/kg dextrose (-□-), and 3 g/kg (-o-) of trial I.

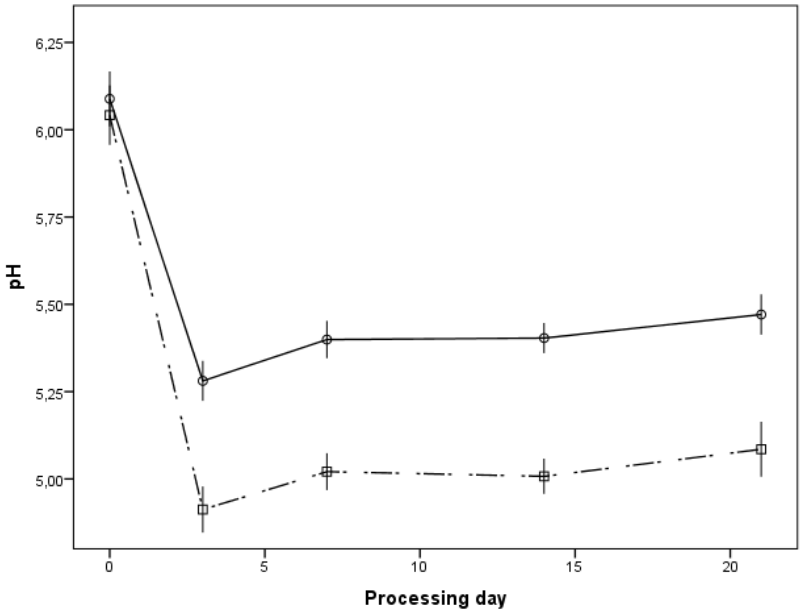


Figure 5.2 pH decrease during the fermentation and ripening stages of sausages prepared with 7g/kg dextrose (-□-), and 3 g/kg (-o-) of trial I.

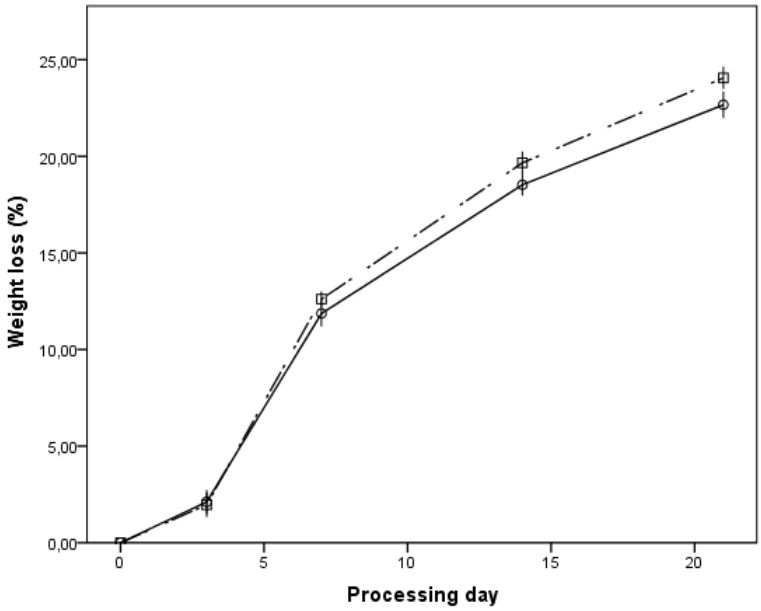


Figure 5.3 Percentage weight loss during the fermentation and ripening stages of sausages prepared with 7 g/kg dextrose (-□-), and 3 g/kg (-○-) of trial I.

5.3.1.2 CONTROL PREPARATIONS

In first instance, dry fermented sausages were prepared without an artificial addition of any precursors (Table 5.1, group A). To investigate the hygienic quality during the production of dry sausages, the biogenic amines contents were analysed. Similar to the results of the samples discussed in Chapter 3 and the biogenic amine contents of sausage types examined in other studies, e.g., the Italian Salsiccia and Soppressata (Parente *et al.*, 2001) and French sausages (Montel, 1999), the studied Belgian type dry fermented sausage model contained mainly TYR, PUT and CAD. In contrast to other studies and the samples analysed in Chapter 3, the evolution of PIP in the meat product was also assessed because of its postulated involvement in the NPIP formation.

No TYR was detected in the freshly made sausages (day 0), but as can be seen in Figure 5.4 A the TYR levels increased over time. Similar to Gençcelep (2007), the omission of $NaNO_2$ in the sausage preparation resulted in significantly higher TYR concentrations. Moreover, in the sausages without added $NaNO_2$, the accumulation of TYR was significantly reduced when the acidification rate was more pronounced. Probably, the explosive growth of the LAB due to the high dextrose dose could compete out the spoilage bacteria in an early stage of the fermentation and thus the enterobacterial decarboxylase enzymes were suppressed (Suzzi & Gardini, 2003). As a result, preparation A1 showed the fastest increase in TYR level due to the missing inhibitory effects of $NaNO_2$ and the higher pH.

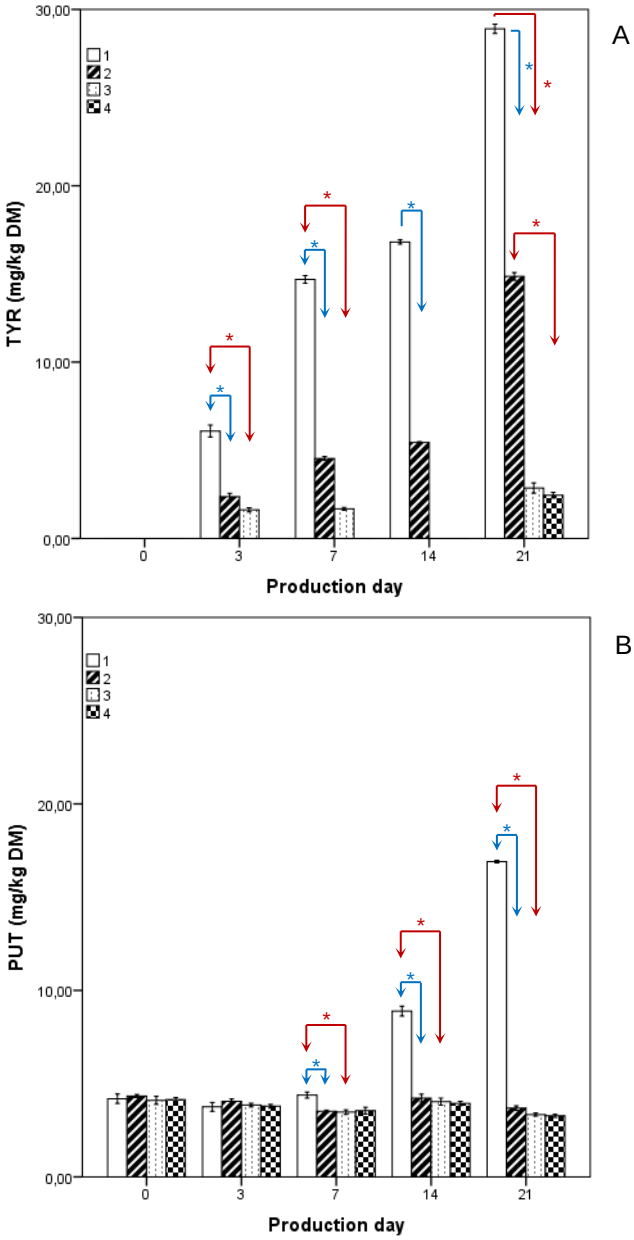


Figure 5.4 Accumulation of TYR (A), PUT (B) during the production of the control dry fermented sausages (group A) of trial I; explanation of the sample codes as in Table 5.1: (1) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 3 g/kg dextrose, (2) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 7 g/kg dextrose, (3) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 3 g/kg dextrose, and (4) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 7 g/kg dextrose (n = 3). Within production days, significant effects (* $p < 0.05$) of $NaNO_2$, within pH levels, are indicated in red and significant effects (* $p < 0.05$) of pH, within $NaNO_2$ levels, are indicated in blue.

Thereby, the highest concentration, found at the end of production was 29.2 mg/kg DM or 16.6 mg/kg product. This is still acceptable compared to concentrations found in commercial products, i.e., an average of 76 mg/kg and 176 mg/kg in respectively Northern European and Southern European products (Ansorena *et al.*, 2002). Moreover, substantially lower than the TYR levels (up to 411 mg/kg), measured in commercial dry fermented sausages at the end of shelf life (Chapter 3).

In contrast to TYR, which was formed during fermentation and drying, the diamines CAD and PUT were already present in the freshly prepared sausages. Initially, the dry fermented sausages contained an average of 4.2 ± 0.2 mg/kg DM PUT (Figure 5.4 B). No significant effect of pH or $NaNO_2$ or their interaction was observed during the fermentation (day 0 and 3). Moreover, during the ripening, no increase in PUT concentrations could be seen when 150 mg/kg $NaNO_2$ was added or the pH was decreased to 4.9. But from day 7, significantly higher PUT concentrations could be found when the sausages were prepared without $NaNO_2$ and had a pH of 5.3 (preparation A1). The observed effects of $NaNO_2$ and pH were in accordance with the conclusions of Gençcelep (2007) and Bover-Cid *et al.* (2001a).

At day 0, the sausage contained an average of 5.0 ± 0.2 mg/kg DM CAD, which increased further during the processing of the sausages (Figure 5.5 A). Similar to the accumulation of TYR and PUT, a significantly faster increase of CAD was present when no $NaNO_2$ was added to the preparation. However, the effect of pH was opposite to that observed for TYR and PUT: higher CAD concentrations were observed at low pH. In literature it is generally accepted that the simultaneous accumulation of TYR, PUT and CAD in dry fermented sausages is mainly attributed to the presence of *Enterobacteriaceae* (Suzzi & Gardini, 2003). As indicated above, the strongest suppression of the enterobacterial population by the LAB could be expected at the highest dextrose dose, so the higher CAD accumulation at pH 4.9 compared to pH 5.3 is rather surprising.

As can be seen in Figure 5.5 B, the PIP concentration raised slowly during the production from 1.8 ± 0.4 mg/kg DM to an average of 8.3 ± 0.7 mg/kg DM. During the subsequent days of the production, main effects of both pH and $NaNO_2$ were observed. However, less pronounced as seen for the biogenic amines discussed earlier. The sausages produced without $NaNO_2$ showed significantly higher PIP concentration. Compared to TYR and PUT, a similar effect of pH on the PIP formation was observed. The addition of extra dextrose, and thus a lower pH of the sausages, significantly reduced the accumulation of PIP. Since fresh meat does not contain detectable amounts of PIP (Bellatti & Parolari, 1982), the presence in the dry fermented sausages could on the one hand be derived from the conversion of CAD (Nikolov & Yaylayan, 2010). On the other hand, it could be attributed to the cleavage of piperine, the alkaloid responsible for the pungency of pepper (Shenoy & Choughuley, 1999).

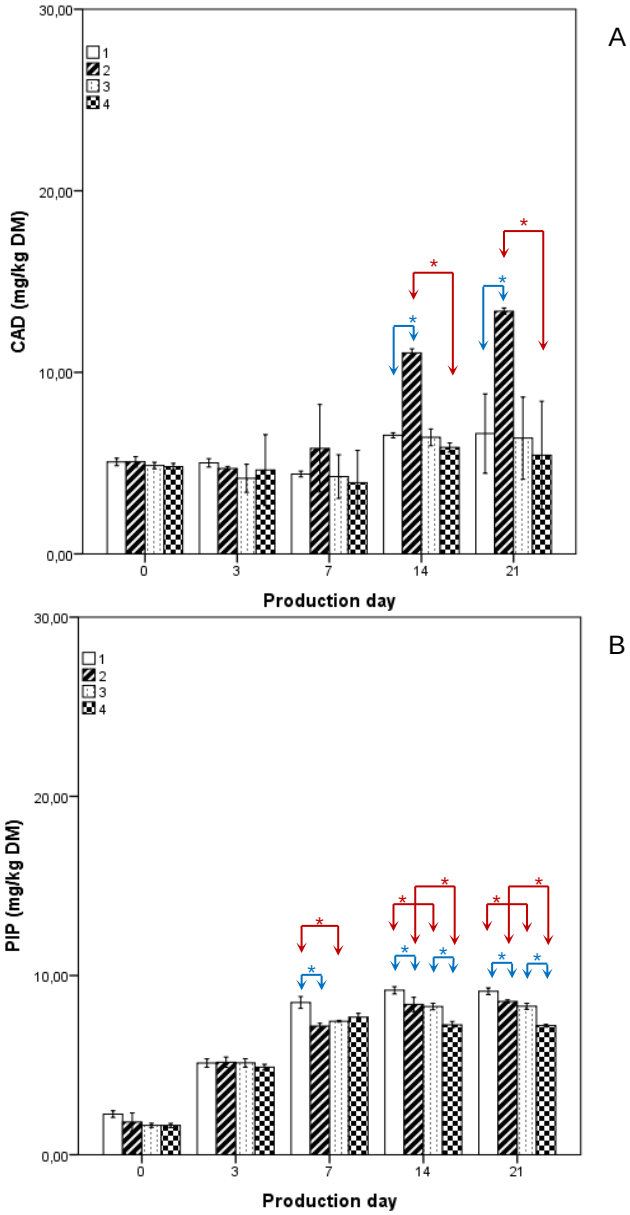


Figure 5.5 Accumulation of CAD (A), PIP (B) during the production of the control dry fermented sausages (group A) of trial I; explanation of the sample codes as in Table 5.1: (1) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 3 g/kg dextrose, (2) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 7 g/kg dextrose, (3) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 3 g/kg dextrose, and (4) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate + 7 g/kg dextrose (n = 3). Within production days, significant effects (* $p < 0.05$) of NaNO2, within pH levels, are indicated in red and significant effects (* $p < 0.05$) of pH, within $NaNO_2$ levels, are indicated in blue.

Concerning *N*-nitrosamine formation, no NDEA or NDBA were detected during the production when there was no artificial enrichment of biogenic amines. Only in a few cases NDMA, NPIP, NPYR and NMOR were detected, but the contamination levels remained almost always under the MQL. Despite the fact that preparations A3 and A4 were made with the nitrosating agent sodium nitrite, no elevated *N*-nitrosamine contamination degree was observed. This means that under controlled hygienic circumstances, dry fermented sausages can be produced without *N*-nitrosamine formation. Even when nitrite was used and the pH was varied from 5.3 to 4.9, the risk could not be induced in the dry fermented sausage model.

5.3.1.3 ADDITION OF PRECURSORS

In the paragraph above, it was concluded that dry fermented sausages could be produced without a meaningful formation of *N*-nitrosamines when they were made under Good Manufacturing Practices (GMP). More specifically, good hygienic circumstances ruled out severe biogenic amine accumulation. However, it has been observed that *N*-nitrosamines, and especially NPIP, can occur in commercial dry fermented sausages (Chapter 3). Shalaby (1996) noted that the biogenic amine CAD, after conversion to PIP, can react with nitrite to form NPIP. In order to evaluate the NPIP formation from the presence of high concentrations of biogenic amines, dry fermented sausages were made with an artificial enrichment of possible precursors. On the one hand CAD.2HCl (500 mg/kg) was added. The intention is to simulate the use of putrified raw meat materials for the production of dry fermented sausages. On the other hand, sausages were prepared with the addition of the direct precursor PIP (10 mg/kg). The concentration approached the PIP content that can be expected upon the extra addition of circa 2 g/kg black pepper in the sausage preparation (Culbreth, 1996).

In Table 5.2, the concentrations of CAD and PIP of the fortified preparations (group B: 500 mg CAD.2HCl, group C: 10 mg/kg PIP) of trial I are given. When 500 mg CAD.2HCl per kg fresh meat batter was added (B), an average of 738 ± 5 mg/kg DM CAD was found in the sausages at day 0. This approached the expected calculated amount of 292 mg/kg CAD or 730 mg/kg DM artificially added on top of the naturally occurring amount of CAD in the fresh sausage batter. After the production (day 21), a substantial reduction of the CAD concentrations were observed. Hereby, the decrease was greater at the lowest pH and when no $NaNO_2$ was added to the meat product. The decrease of CAD can be explained by the presence of amine oxidases which can catalyse the deamination of biogenic amines (Gardini *et al.*, 2002). Also the PIP concentration of the CAD enriched sausages were significantly higher than could be naturally found in the control samples. However the increased PIP formation in the presence of the excessive amount of CAD occurred mainly in the freshly stuffed sausages. During the production, only a small amount of PIP was additionally formed.

Table 5.2 Detected CAD and PIP concentrations (mg/kg DM) during the production of dry fermented sausages artificially enriched with CAD (group B: 500 mg/kg CAD.2 HCl) and PIP (group C: 10 mg/kg PIP) in trial I.

			Production day*			
			CAD		PIP	
Precursor	$NaNO_2$	pH	0	21	0	21
CAD (B)	0	5.3	$735 \pm 5^{b,1}$	$685 \pm 3^{a,2}$	$12.6 \pm 0.3^{a,1}$	$14.7 \pm 0.2^{b,2}$
		4.9	$741 \pm 4^{b,1}$	$640 \pm 3^{a,1}$	$12.0 \pm 0.1^{a,1}$	$13.0 \pm 0.0^{a,1}$
	150	5.3	$736 \pm 2^{b,1}$	$705 \pm 3^{a,2}$	$12.7 \pm 0.1^{a,1}$	$14.4 \pm 0.1^{b,2}$
		4.9	$741 \pm 9^{b,1}$	$663 \pm 3^{a,1}$	$12.8 \pm 0.2^{a,1}$	$12.6 \pm 0.1^{a,1}$
PIP (C)	0	5.3	$5.6 \pm 0.6^{a,1}$	$9.3 \pm 0.3^{b,2}$	$19.1 \pm 0.2^{a,1}$	$22.4 \pm 0.1^{b,2}$
		4.9	$6.5 \pm 0.3^{a,1}$	$9.7 \pm 0.1^{b,2}$	$21.7 \pm 0.2^{a,1}$	$21.8 \pm 0.1^{a,2}$
	150	5.3	$5.3 \pm 0.5^{b,1}$	$3.5 \pm 0.3^{a,1}$	$19.6 \pm 0.1^{a,1}$	$20.3 \pm 0.1^{b,1}$
		4.9	$4.6 \pm 0.3^{b,1}$	$2.7 \pm 0.1^{a,1}$	$20.0 \pm 0.1^{a,1}$	$19.9 \pm 0.1^{a,1}$

*mean ± standard deviation (n = 3). Within added precursor, different letters in the same row indicate significant differences ($p < 0.05$) in CAD or PIP content. Within added precursor (B, C), different numbers in the same column indicate significant differences ($p < 0.05$) in CAD or PIP content.

Initially the addition of 10 mg/kg PIP (group C) did not influence the CAD levels, but at the end of production (day 21), the CAD levels of the PIP fortified samples were suppressed by the addition of sodium nitrite to the sausage model. It seems that the combined antibacterial effect of sodium nitrite and PIP can inhibit the formation of CAD during the processing. As intended, the highest PIP concentrations were measured in the PIP enriched sausages (group C). Although the PIP content increased further during the production, the growth was minute compared to the control samples. Thereby, the addition of sodium nitrite resulted in a slightly, yet significantly lower concentration at day 21, but no significant influence of the pH was observed.

In this study, no other volatile *N*-nitrosamines, besides NPIP, were detected during the production of the dry fermented sausage preparations. The range of NPIP concentrations of the first trial are given in Table 5.3. For a statistical analysis of data sets (group A) and (group B), the number of samples with NPIP > MDL was too small. Both groups, i.e., control and CAD enriched sausages are mostly free of NPIP contamination. It can be concluded that the role of CAD in the NPIP formation is negligible under the investigated GMP conditions of the dry fermented sausage model production. For the NPIP formation, only the results of data set (group C) could be evaluated, indicating the relevance of elevated PIP in the formation of NPIP. The three way ANOVA, with variable factors production day (x_1), added $NaNO_2$ concentration (x_2), and pH (x_3), revealed a main effect of the added sodium nitrite concentration on the formation of NPIP. In contrast to the inhibitory effect of $NaNO_2$ on the biogenic amine accumulation, the presence of nitrite enhances the *N*-nitrosamine formation. However, the NPIP contamination was not influenced by differences in final pH. Moreover, no significant differences between time points were observed. It means that no augmentation in NPIP concentration was seen during the production.

Secondly, it seems that nitrite is the major supplier of the nitrosating agent since the formation of *N*-nitrosamines occurs mainly at the beginning of the production, when still an adequate amount of $NaNO_2$ is present. As mentioned above, in the presence of sodium ascorbate, the residual $NaNO_2$ level drops very fast (Honikel, 2008) and thus almost no $NaNO_2$ is left to be involved in the *N*-nitrosamine formation during the production.

Table 5.3 Range of the detected NPIP concentrations (µg/kg DM) during the production of the dry fermented sausage models in trial I.

			Production day[a,b]				
Precursor	$NaNO_2$	pH	0	3	7	14	21
Control	0	5.3	nd	nd	nd - 1.8	nd	nd
(A)		4.9	nd	nd	nd	nd	nd
	150	5.3	nd - 1.2	nd	nd	nd	nd
		4.9	nd	nd - 3.7	nd - 1.0	nd - 1.0	nd
CAD	0	5.3	nd	nd	nd	nd	nd
(B)		4.9	nd	nd	nd - 1.4	nd	nd
	150	5.3	nd	nd	nd	nd	nd
		4.9	nd	nd	nd	nd	nd
PIP	0	5.3	nd	nd - 1.7	nd	nd - 1.0	nd
(C)		4.9	nd	nd	nd - 1.5	nd - 0.9	nd - 1.6
	150	5.3	1.2 - 1.8	nd - 1.3	1.0 - 2.9	nd - 0.9	nd - 1.0
		4.9	nd - 1.1	nd	0.9 - 1.6	nd	nd

[a]nd: not detected, [b]min – max (n = 3).

5.3.2 TRIAL II

5.3.2.1 PRODUCTION MONITORING

In the first trial, it was concluded that PIP could be a precursor of NPIP formation in dry fermented sausages, therefore a second trial was carried out to investigate the influence of the nitrosating agent on the NPIP formation when the artificially added PIP concentration was varied (see Table 5.1).

In this second trial, dry fermented sausages, enriched with PIP, were made with and without sodium nitrite and with and without the addition of sodium ascorbate. In this way the availability of the nitrosating agent during the production could be varied. In Figure 5.6, the decline of the nitrite concentration during the production is given. At day 0, a significant difference in residual $NaNO_2$-level was observed. As could be expected, no nitrite could be detected in the sausages in which no sodium nitrite was added (code 2 in Table 5.1). In the sausages prepared with 150 mg/kg $NaNO_2$ (see codes 4 and 5 in Table 5.1), residual levels can be detected. But in presence of sodium ascorbate, immediately after stuffing (day 0), the concentration dropped below the quantification limit (MQL = 10 mg/kg). If sodium ascorbate was omitted (code 5 in Table 5.1) in the sausage preparations, an average level of 37 ± 14 mg/kg DM $NaNO_2$ was noted at day 0, which means that only 10% or 16 ± 6 mg/kg of the added 150 mg/kg $NaNO_2$ was left after stuffing. Nevertheless, the difference was

limited to day 0. From day 3 until the end of production, the residual $NaNO_2$ levels found in all the variations dropped below the MQL.

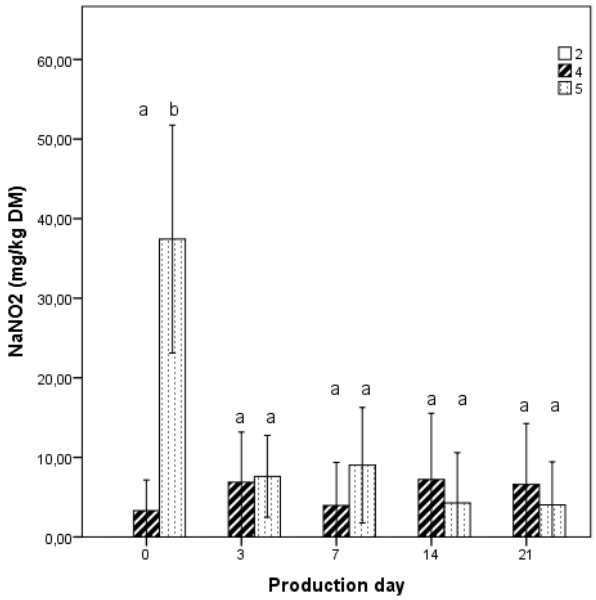


Figure 5.6 Sodium nitrite concentration during the production process of dry fermented sausages of trial II, explanation of the sample codes as in Table 5.1: (2) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate, (4) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate, and (5) 150 mg/kg sodium nitrite + 0 mg/kg sodium ascorbate (n = 3). Bar charts with different superscript are significantly different at $p < 0.05$ within a production day.

5.3.2.2 FORMATION OF NPIP

In contrast to the first trial, NPIP concentrations were in general higher, ranging from not detected to a maximum of 18.1 µg/kg. At day 0 (Figure 5.7 A) the addition of sodium nitrite (codes 4 and 5 in Table 5.1) promotes NPIP formation at both PIP levels. Omission of sodium ascorbate in the preparations (code 5 in Table 5.1) gave only significantly increased NPIP concentrations at the highest PIP level. Earlier in this study it was illustrated that the degradation of nitrite was enhanced by the use of sodium ascorbate. Hereby, the role of the residual nitrite concentration in the *N*-nitrosamine formation in meat products is confirmed. Although the usefulness of sodium ascorbate is proven, the effect was limited to the fresh meat batter (day 0). Once the dry fermented sausages were ready for consumption at day 21 the NPIP concentrations were reduced and the effect of the nitrosating agent had disappeared (Figure 5.7 B). *N*-nitrosamines can be reduced by hydrophotolysis (Ahn *et al.*, 2003). However the occurrence of this reaction in the dry fermented sausage production was most unlikely since the necessary water content and light source were lacking. More likely, as suggested by Hauser and Heiz (1978), an enzymatic breakdown attributed

to the microbial activity during the ripening of the sausage can have occurred.

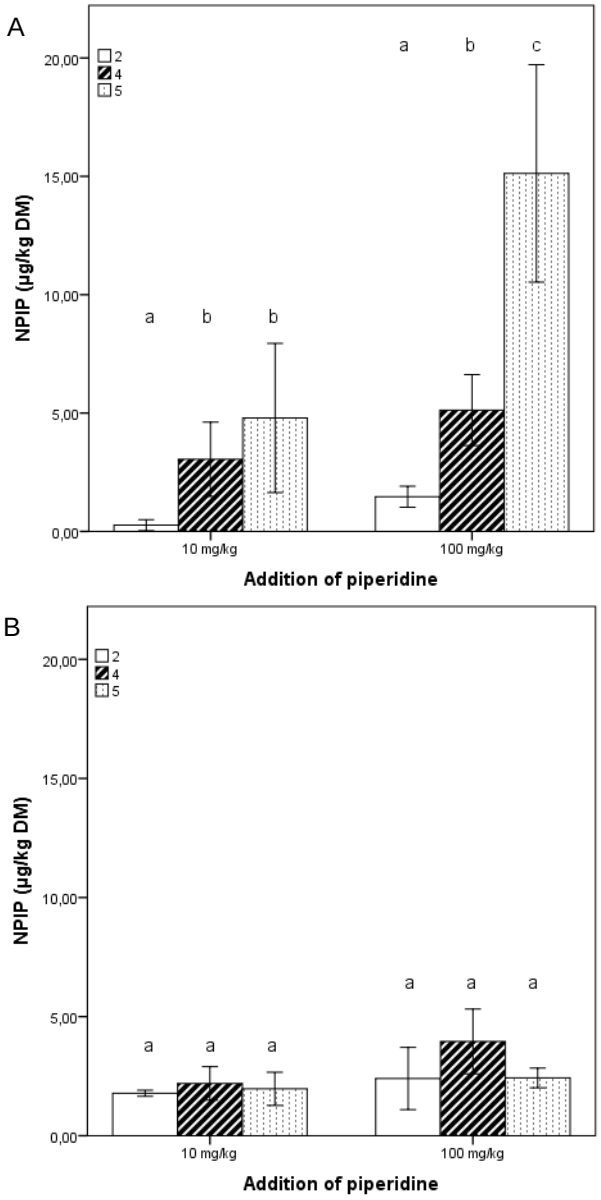


Figure 5.7 NPIP concentrations in the dry fermented sausages at day 0 (A) and day 21 (B) of trial II; explanation of the sample codes as in Table 5.1: (2) 0 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate, (4) 150 mg/kg sodium nitrite + 500 mg/kg sodium ascorbate, and (5) 150 mg/kg sodium nitrite + 0 mg/kg sodium ascorbate (n = 3). Bar charts with different superscripts are significantly different at $p < 0.05$ (Duncan's post hoc test) within PIP concentration.

5.4 CONCLUSIONS

The analyses of the biogenic amines of the control preparations demonstrated that the accumulation of the major biogenic amines, i.e., TYR, PUT and CAD could be inhibited by the use of 150 mg/kg sodium nitrite in the sausage preparation. Furthermore, even when no sodium nitrite was added, the biogenic amine content remained low during the whole production. Moreover, none of the variations of the control samples showed a distinct *N*-nitrosamine formation.

The artificial enrichment with a large amount of CAD did not invoke any accumulation in the formation of *N*-nitrosamines during the fermentation and ripening. In contrast, PIP could act as precursor for the NPIP formation. Thereby, in the pH range of dry fermented sausages, no effect of the acidification degree was observed. The role of residual nitrite level in the formation of *N*-nitrosamines was confirmed, but it should be noted that the use of ascorbate as nitrite scavenging agent had only effect at the beginning of the production. Afterwards the residual nitrite levels of all variations were not high enough to form a risk in the *N*-nitrosamine formation.

CHAPTER 6
STUDY OF THE *N*-NITROSOPIPERIDINE FORMATION IN A PROTEIN-BASED SYSTEM[5]

[5] This chapter was based on following paper:
De Mey, E., Viaene, J., Dejaegher, B., De Maere, H., Dewulf, L., Paelinck, H., Vander Heyden, Y., Fraeye, I. (2013). A study of the effects of pH and water activity on the *N*-nitrosopiperidine formation in a protein-based liquid system, Food and Bioprocess Technology, DOI: 10.1007/s11947-013-1249-7.

6.1 INTRODUCTION

In contrast to cooked meat products, dry sausages undergo a fermentation process causing a decrease of pH from approximately 5.7 to values between 4.5 and 5.5 (Toldrá, 2007). This acidification can promote the *N*-nitrosamine formation since the nitrosation of secondary amines requires an acidic environment (pH < 5) (Challis & Kyrtopoulos, 1977). However, at pH values above 4, the addition of moderate concentrations of sodium chloride (max. 12%) inhibited the formation of *N*-nitrosamines (Hildrum *et al.*, 1975; Theiler *et al.*, 1981; Rywotycki, 2002). The inhibitory effect of sodium chloride has been explained by its contribution to the ionic strength of the medium, which influences the reaction between nitrite and secondary amines (Bulushi *et al.*, 2009). While dry fermented sausages contain a certain amount of sodium chloride (ca. 3%), the product is also characterized by a reduced water activity. The obtained a_w-levels are the result of the combined action of sodium chloride and the removal of water during drying. However, in the literature, no evidence was found that a reduced a_w, apart from the influence of sodium chloride, inhibits the nitrosation reaction.

Response Surface Methodology (RSM), initially described by (Box & Wilson, On the experimental attainment of optimum conditions., 1951) can be used for the modeling and analysis of a response, which is influenced by several variables. Although RSM is mainly used for the optimization of industrial processes (Ahmadi, 2005); Saxena *et al.*, 2009) and analytical procedures (Hossain *et al.*, 2011), it is also applied to study the formation of toxic contaminants in food products, e.g., HAs (Gibis, 2007; Dundar *et al.*, 2012) and acrylamide (Lasekan & Abbas, 2011). The advantage of RSM is the possibility to test several variables simultaneously by means of an experimental design, such as a central composite design (CCD). From the experimental results, a model can be build which estimates the relationship between the variables and the response.

The aim of this chapter is to estimate the influences of pH and water activity on the NPIP formation during the production of dry fermented sausages. For that purpose, two liquid systems were built, in which PIP and sodium nitrite were added. In the first system (NaCl system), at two pH values (pH 4.0 and 5.0), sodium chloride was added to reduce a_w, prior to measuring the NPIP levels after 72 h. The second system contained mixtures of polyethylene glycol (PEG) and water to reduce the water activity. RSM was used to evaluate the influences of pH (3.0 – 7.0), a_w (0.80 – 0.99) and incubation time (1.3 – 98.7 h) on the NPIP concentration in the PEG system.

6.2 MATERIALS AND METHODS

6.2.1 PREPARATION OF THE PROTEIN-BASED SYSTEMS

Two types of liquid systems (25 mL) were prepared in plastic test tubes (VWR International, West Chester, PA, USA). The first system (NaCl system) consisted of brain-heart-infusion broth (BHI, 37 g/L, Merck, Darmstadt, Germany) dissolved in water. In order to reduce the water activity, sodium chloride (VWR International) was added to the test tubes in the range of 0 to 30 g/100 mL, resulting in a_w values between 0.990 and 0.790, respectively. The test tubes were sterilised in a bench-top autoclave (Systec D-150, Wettenberg, Germany) and after cooling, PIP (100 mg/L) was added. The initial pH of the mixture (pH 7.5) was reduced by the addition of 300 and 450 µl lactic acid (90%, VWR International) in order to obtain pH values of 5 and 4, respectively. Finally, sodium nitrite (150 mg/L, VWR International) was added just before incubation at 26 °C for 72 hours. Experiments using the NaCl system were done in quadruple.

The second system (PEG system) was prepared by dissolving BHI (37 g/L) in a mixture of water and polyethylene glycol 200 (PEG, Merck). In order to reduce the water activity, the ratio of PEG : water was varied, ranging from 0:25 (v/v) to 15:10 (v/v), resulting in a_w values between 0.990 to 0.800. Similar to the NaCl system, the test tubes were sterilised and PIP and sodium nitrite were added. The pH of the samples was altered by the addition of 200 - 900 µl lactic acid (90%), to cover the pH range of 3.0 - 7.0. The levels of a_w and pH of the PEG systems, according the experimental design (as described below), are given in Table 6.1.

Table 6.1 Independent factors and their levels for the CCD. Experimental set-up of the PEG system.

Factor	Symbol	Factor coding				
		-1.68	-1	0	+1	+1.68
pH	x_1	3.0	3.8	5.0	6.2	7.0
a_w	x_2	0.800	0.839	0.895	0.952	0.990
time (h)	x_3	1.3	21.0	50.0	79.0	98.7

6.2.2 PHYSICAL AND CHEMICAL ANALYSES

The pH was measured by immersing the glass pH electrode (KnickPortamess, Elscolab, Terschuur, The Netherlands) in the liquid samples. The water activity (a_w) was determined using a dewpoint hygrometer (Aqualab, Decagon Devices, Pullman, USA).

The NPIP content was determined according to the *N*-nitrosamine method of Drabik-Markiewicz *et al.* (2011), as described in Chapter 3.2.3.3. The method was modified for analyses of liquid samples. Therefore, 25-mL aliquots of the liquid systems were used instead of 50-g solid samples. The measured concentrations were expressed as µg/mL.

6.2.4 RESPONSE SURFACE DESIGN

The formation of NPIP in the PEG system was studied using RSM. A three-factor, five-level Rotatable Central Composite Design (RCCD) (Box *et al.*, 1978) was used. The factors and their levels are given in Table 6.1. The design consisted of 15 experiments (Table 6.2), and was executed in triplicate. To estimate the response surface, the experimental data were fitted to a quadratic polynomial equation:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (1)$$

in which the NPIP concentration (response Y) was correlated to the factors pH (x_1), a_w (x_2), and time (x_3), and with b_0 the intercept, b_1, b_2 and b_3 the linear, b_{12}, b_{13}, and b_{23} interaction and b_{11}, b_{22}, and b_{33} the quadratic coefficients. The model was evaluated by the Fisher test value (F-value), the coefficient of multiple determination (R^2) and the adequacy (paired t-test of the experimental and fitted predicted data).

6.2.5 DATA ANALYSIS

In the NaCl system, the influences of the independent variables, NaCl concentration and pH, on the NPIP formation were evaluated by a two-way ANOVA, with the Tukey's honestly significant difference criterion as post hoc test. A three-way ANOVA was used for the PEG system (independent variables pH, a_w and incubation time). For all statistics, significance was determined at the 5% significance level ($\alpha = 0.05$). The software Matlab 5.3 (The Math-Works, Natick, MA, USA) and PASW Statistics 20.0.0 (SPSS, Armonk, NY, USA) was used for all statistical and graphical analyses.

6.3 RESULTS AND DISCUSSION

6.3.1 INFLUENCE OF A_W AND PH ON THE NPIP FORMATION IN THE NACL SYSTEM

During the production of dry fermented sausages, a drying step is included to achieve the desired water activity and flavour characteristics. Depending on the drying period and the sausage diameter, the water activity of the sausages usually decreases from 0.96 to 0.82 - 0.90 (Toldrà, 2002). In the NaCl system, the water activity was reduced from 0.990 to 0.790 by the addition of increasing concentrations of sodium chloride (0 - 30%) (Table 6.3).

Table 6.2 Central composite design, performed on the PEG system, to study the influence of three factors on the NPIP concentrations.

Exp. no.	Factor level			NPIP (µg/ml)[a]		
	pH (x_1)	a_w (x_2)	time (x_3)	Y_{exp}[b]	Y_{pred}[c]	Y_{exp}-Y_{pred}[d]
1	-1	-1	-1	36.3±1.4	30.8	5.5±1.5
2	+1	-1	-1	9.9±0.4	5.8	4.1±0.4
3	-1	+1	-1	61.6±5.3	59.1	2.5±5.3
4	+1	+1	-1	12.3±7.0	20.0	-7.7±7.0
5	-1	-1	+1	94.0±7.1	78.6	15.4±7.1
6	+1	-1	+1	33.9±2.4	28.6	5.2±2.4
7	-1	+1	+1	113.6±0.7	110.0	3.6±0.7
8	+1	+1	+1	48.2±2.5	45.9	2.2±2.5
9	-1.68	0	0	63.4±8.1	75.8	-12.4±8.1
10	+1.68	0	0	2.5±0.7	1.0	1.5±0.7
11	0	-1.68	0	16.0±1.3	30.3	-14.2±1.3
12	0	+1.68	0	71.9±2.1	68.6	3.3±2.1
13	0	0	-1.68	21.1±5.0	20.0	1.1±5.5
14	0	0	+1.68	69.9±8.4	81.9	-12.0±8.4
15	0	0	0	30.6±5.7	28.7	1.9±5.7

[a]Mean±stdev (n=3), [b]experimental value, [c]predicted value; [d]residue.

Table 6.3 NPIP formation (µg/mL) at different pH after 72 hours incubation, influenced by the NaCl concentration of the NaCl system.

Added NaCl (g/100mL)	a_w	NPIP	
		pH 4.0	pH 5.0
0	0.990	$30.8 \pm 2.1^{a,1}$	$18.0 \pm 2.5^{b,1}$
10	0.935	$28.1 \pm 1.3^{a,1}$	$17.3 \pm 0.7^{b,1}$
20	0.868	$7.1 \pm 0.6^{a,2}$	$17.7 \pm 2.2^{b,1}$
30	0.790	$6.2 \pm 0.2^{a,2}$	$11.5 \pm 0.8^{b,2}$

Different letters (a, b) in the same row indicate significant differences ($p < 0.05$) among the pH levels. Different numbers (1, 2) in same column indicate significant differences ($p < 0.05$) among the NaCl concentrations. Data are given as mean ± standard deviation (n = 4)

At a given a_w value the NPIP concentrations at pH 4.0 and 5.0 were always found to be different. Both at pH 4.0 and 5.0, a significant effect of a_w, caused by the varying sodium chloride concentrations, is also seen on the NPIP formation. At pH 4.0, high NPIP levels were measured at a_w levels of 0.935 or higher, which is corresponding with a concentration of 10% NaCl or lower. When the NaCl concentration increased (to 20% or more) and thus the a_w was reduced to 0.868 or lower, the NPIP concentrations decreased significantly. Compared to the results obtained at pH 4.0, the NPIP formation at pH 5.0 was significantly lower at high a_w values. The NPIP concentrations were again reduced by increasing salt concentrations, but the effect was less pronounced at pH 5.0. As a result, at reduced a_w-levels, the NPIP concentrations were higher at the highest investigated pH. Previously, an inhibitory effect of NaCl (up to 12%) on *N*-nitrosamine formation has been reported (Hildrum *et al.*, 1975; Theiler *et al.*, 1981; Rywotycki, 2002). Our results confirm this observation. However, it is important to note that it is unclear to what extent the observed effects can be attributed to an increase in ionic strength, or a reduction in a_w. In dry fermented sausages, the low a_w value results from, on the one hand a low amount of NaCl (ca. 3%) and on the other hand removal of water. The high NaCl concentrations (up to 30%) used in the model system, in order to achieve the low a_w levels, do not reflect conditions in dry fermented sausages. Therefore, in order to study only the effect of reducing the water activity in dry fermented sausages and avoiding extreme ionic effects of salts, as sodium chloride, an alternative additive, namely PEG, was used.

6.3.2 Influence of pH, A_W and time on the NPIP formation in the PEG system

Because of its good water solubility and stability to acid and high temperatures (Chen *et al.*, 2005), PEG is often used to reduce the water activity in experimental models (Hallsworth & Magan, 1999; Martinez *et al.*, 2001). Although an inhibitory effect of PEG on colon cancer, initiated by *N*-nitrosamines and HAs, was demonstrated by Corpet *et al.* (2000), the choice of low molecular poly ethylene glycol (PEG 200) to reduce the water activity of the liquid model (PEG system) is justifiable since the anti-tumor

effects are only related to high molecular PEGs, such as PEG 8000, while no chemical properties, which can influence the nitrosation reaction are known for low molecular PEGs, such as PEG 200.

In this experiment, the formation of NPIP was studied as a function of pH, water activity and time. For the purpose of identifying the region with the highest NPIP formation, a quadratic model was applied. The experimental data, obtained from a RCCD set-up, are shown in Table 6.2 and allowed the development of a quadratic model (eq. (1)), where the NPIP concentration (Y) is expressed as a function of pH (x_1), a_w (x_2) and time (x_3). The resulting model is as follows:

$$Y = 28.66 - 22.25x_1 + 11.40x_2 + 18.42x_3 - 3.53x_1x_2 + 6.24x_1x_3 + 0.78x_2x_3 + 3.45x_1^2 + 7.36x_2^2 + 7.90x_3^2 \quad (2)$$

The regression model was highly significant (F = 43.6, $p < 0.001$) and the total variance was highly explained ($R^2 = 0.918$). The adequacy of the model was checked by the comparison of the experimentally obtained and the predicted values. The experimental (Y_{exp}) and predicted values (Y_{pred}), together with the residues (Y_{exp} - Y_{pred}) are given in Table 6.2. As no significant difference was found between the experimental and predicted values, the adequacy of the model was confirmed. Therefore, the model can be employed for the description of NPIP formation in the liquid protein-based system.

In order to easily interpret the effects and interactions of the variables, as statistically analysed by ANOVA (Table 6.4), the model (Eq. 2) was visualized by response surface plots and contour plots (Figure 6.1 – 6.3). Whereby the contour plot represent a 2-D projection of the corresponding 3-D surface plot. However, one should realize that these plots only visualize a small part of the entire response surface, which is situated in a four-dimensional space. To visualize, one factor is to be kept constant. For this factor the response surfaces at -1.68, 0 and +1.68 were evaluated and a similar behavior was seen. Therefore, the surfaces and contours at levels 0 were plotted and discussed.

Table 6.4 Results of the three-way ANOVA analyses on the NPIP concentrations, with x_1 (pH), x_2 (a_w), x_3 (time). Bold: significant ($p < 0.05$).

Factor	F	*p*
x_1	**202**	**0.000**
x_2	**63**	**0.000**
x_3	**144**	**0.000**
x_1x_2	**8.7**	**0.006**
x_1x_3	**27**	**0.000**
x_2x_3	0.43	0.517
$x_1x_2x_3$	3.3	0.077

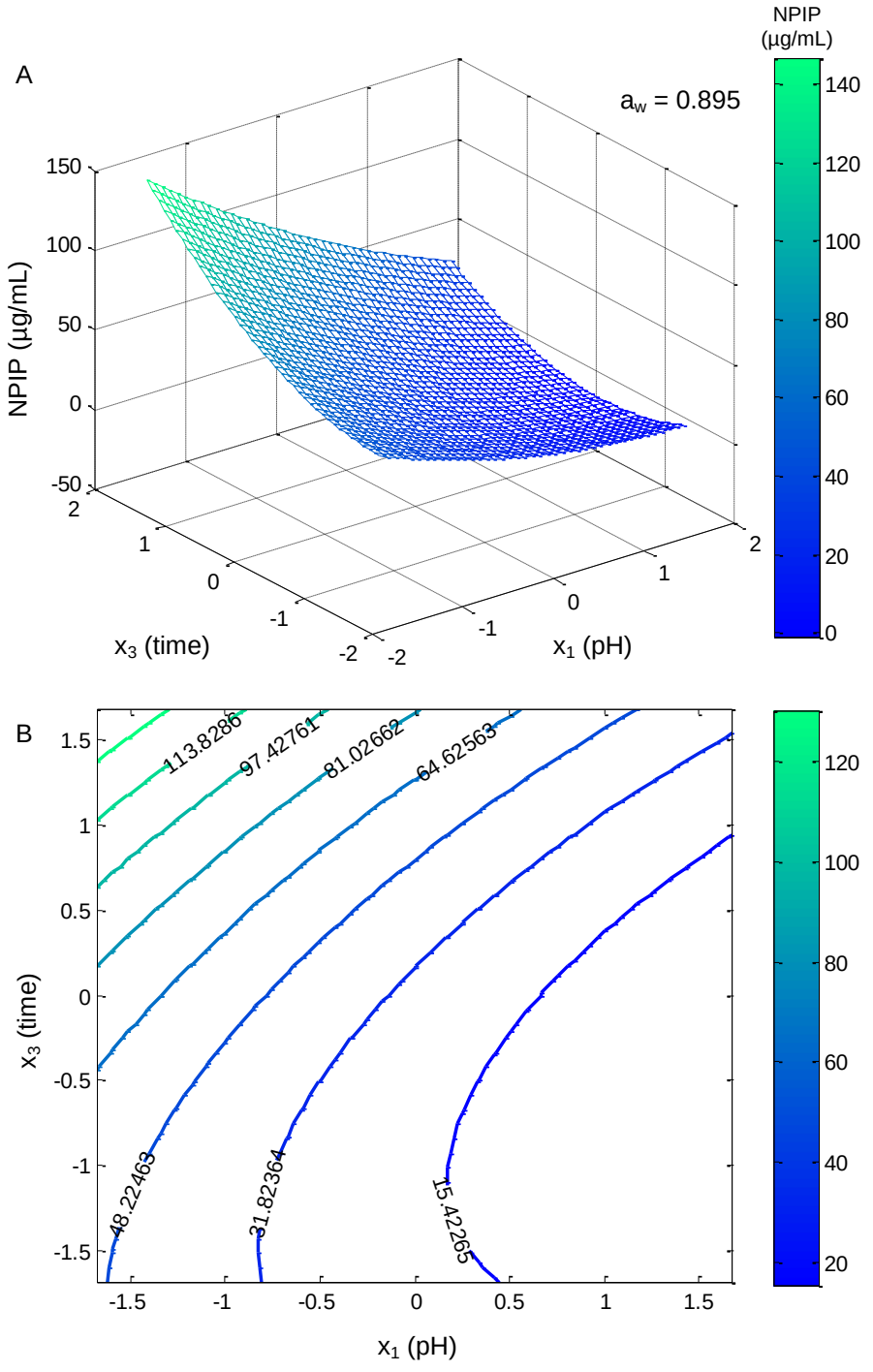


Figure 6.1 Response surface plot (A) and the corresponding 2-D projection on the contour plot (B), representing the response (NPIP concentration) in the PEG system for the effects of pH (x_1) and time (x_3) at an a_w (x_2) level 0 (0.895).

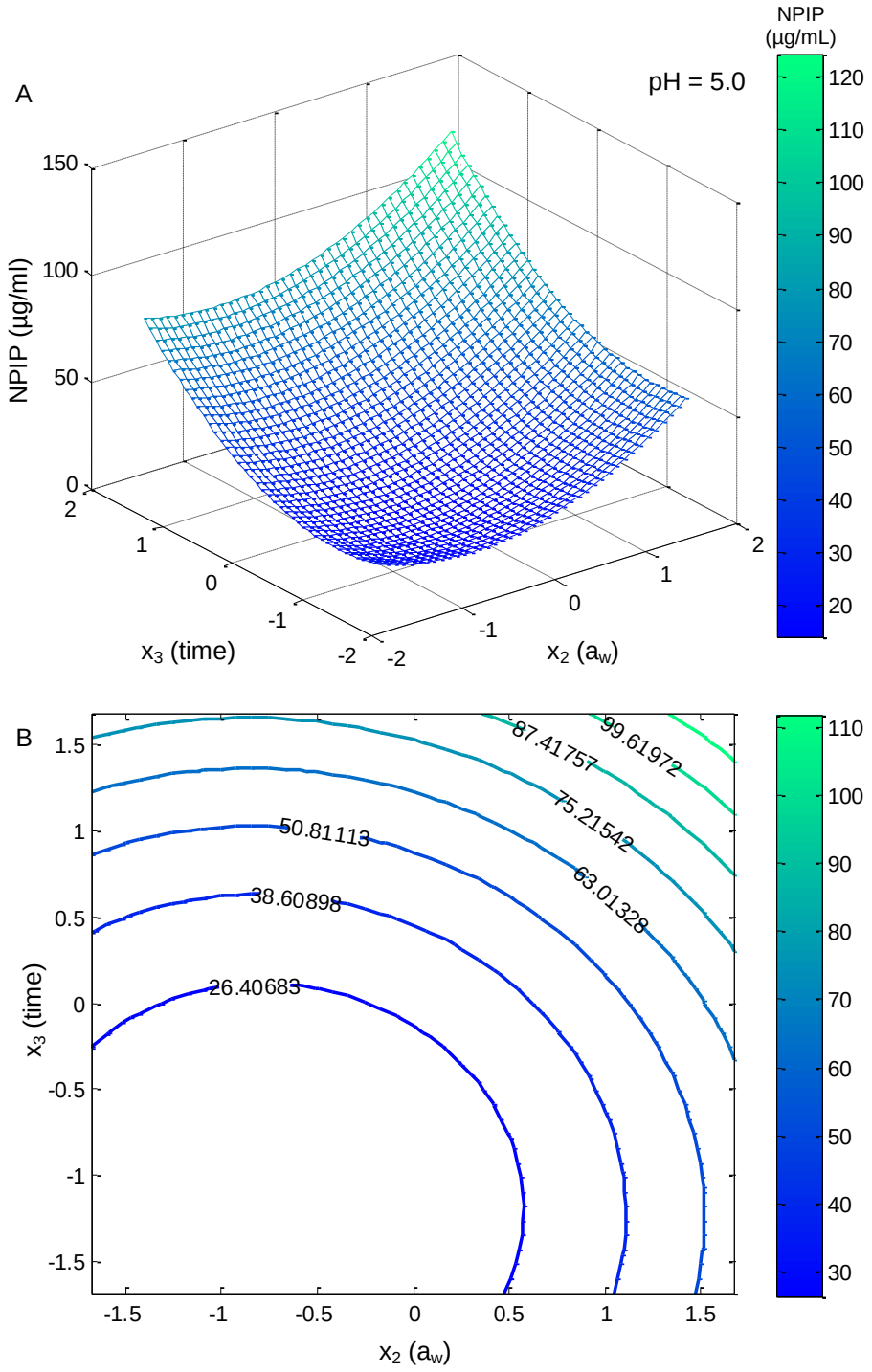


Figure 6.2 Response surface plot (A) and the corresponding 2-D projection on the contour plot (B), representing the response (NPIP concentration) in the PEG system for the effects of a_w (x_2) and time (x_3) at a pH (x_1) level 0 (5.0).

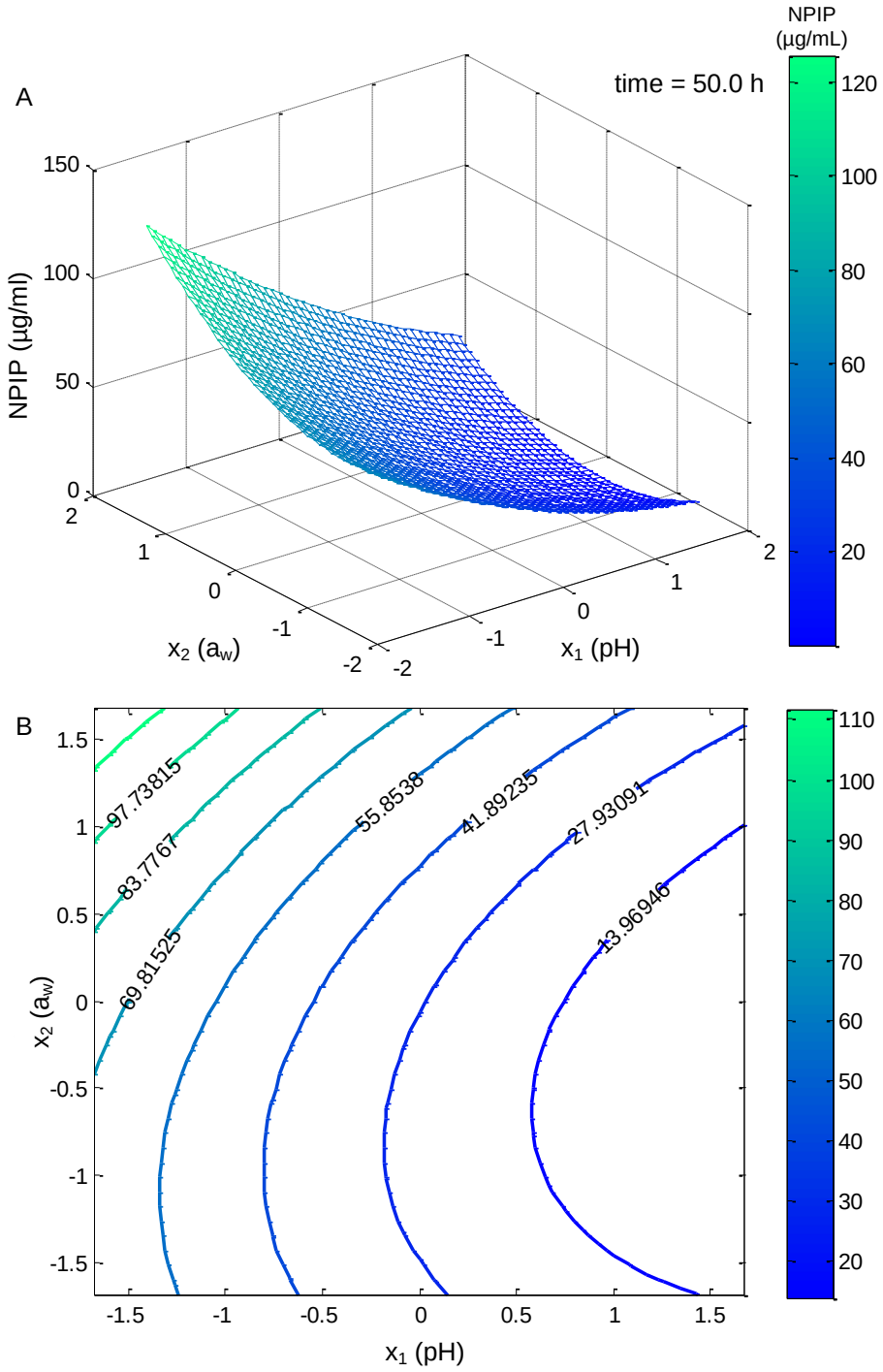


Figure 6.3 Response surface plot (A) and the corresponding 2-D projection on the contour plot (B), representing the response (NPIP concentration) in the PEG system for the effects of pH (x_1) and a_w (x_2) at the time (x_3) level 0 (50 h).

In Figure 6.1, the response surface plot and contour plot are given which represent the effect of pH (x_1) and time (x_3) at a constant a_w of 0.895 ($x_2 = 0$). It seems evident that a longer incubation time, resulted in higher NPIP formation. Moreover, a decrease of pH significantly increased the NPIP concentrations. As a consequence, maximum NPIP formation was detected at the lowest pH level of the design and the highest time. This observation is in accordance to the study of Mirvish (1975), which situated the pH optimum for the nitrosation of PIP at ca. pH 3.0. Nevertheless, it is important to note that the solutions used in the PEG system were sterilised before incubation, preventing biological degradation of the *N*-nitrosamines formed. In contrast, degradation of NPIP was observed in the dry fermented sausage model preparations studied in Chapter 5, which can probably be attributed to microbial activity in the meat products (Hauser & Heiz, 1978).

In Figure 6.2, the effects of a_w (x_2) and time (x_3) are presented at pH 5.0 ($x_1 = 0$). The highest NPIP concentrations were observed after the longest incubation time ($x_3 = 1.68$), which was more than four days, and at the highest water activity ($x_2 = 1.68$). In other words, a decrease of the water activity of the system, by means of increasing amounts of PEG, significantly inhibited the formation of NPIP. Moreover, the inhibitory effect of decreasing a_w was less pronounced at a_w values below 0.895 ($x_2 = 0$) because in that domain the NPIP formation was already low. Since no effects of PEG on the *N*-nitrosamine formation are known, it can be assumed that only the water activity influenced the nitrosation reaction of PIP. As a consequence, the inhibition of *N*-nitrosamine formation in food products is presumably not only attributed to the ionic effects of the added chloride. As shown in the PEG system, decreasing the water activity, without increasing the NaCl concentration, resulted also in an inhibitory effect on the *N*-nitrosamines formation. It thus can be concluded that the drying step in the production of dry fermented sausage is not only enhancing the microbial safety but also can be considered important in the inhibition of NPIP.

Figure 6.3 represents the response surface plot and contour plot where the effects of pH (x_1) and a_w (x_2) on the NPIP concentration (Y) at constant incubation time of 50 h ($x_3 = 0$) are given. As already discussed, higher NPIP concentrations can be observed when the pH decreases and water activity increases. The highest NPIP concentrations can be found in the system with pH 3.0 ($x_1 = -1.68$) and a_w 0.990 ($x_2 = 1.68$). Moreover an interaction (Table 6.4) between pH and a_w occurred. The effect of a pH decrease was larger when the water activity was high, while the NPIP concentrations during the acidification of an environment with low a_w increased less rapidly (Figure 6.3). Similar effects were seen in the NaCl system, although there, the NPIP concentrations at high pH and low a_w were higher than at low pH and low a_w (disordinal interaction). Here the interaction effects between a_w and pH was much more important (Table 6.3).

From the above discussion and from the prediction of grid points (Figure 6.4) it is observed that the highest NPIP concentrations are predicted at low pH-values and at high values for a_w and time.

6.3.3 IMPLICATIONS FOR THE SAFETY OF DRY FERMENTED SAUSAGES

In this study, the systems were all prepared with the addition of 100 mg/L PIP and 150 mg/L sodium nitrite. Consequently, since equimolar amounts of both precursors are necessary to form NPIP, PIP was the limiting precursor. As a result, a maximum of 134 µg/mL NPIP could be formed in the system when a 100% conversion would occur. Within the experimental domain of the design a maximum of 110.0 µg/mL (or a yield of 82%) was predicted after an incubation time of 79 h at a pH of 3.8 and an a_w-value of 0.952. As can be seen in Table 6.2, the experimental maximum of 113.6 µg/mL NPIP was measured at the same conditions. Fortunately, such high yields do not occur in dry fermented sausages. Firstly, the amount of PIP will be less since it is mainly introduced by the addition of pepper in the sausage. Approximately 22 mg PIP may be present in one kilogram of meat batter since 2 g pepper, containing ca. 11 mg PIP per gram pepper, is used for the preparation of dry fermented sausages (see Chapter 7). Secondly, the added amount of sodium nitrite, legally restricted to max. 150 mg per kg meat product (Directive 2006/52/EC), will firstly react with various compounds of the meat such as heme, sulfhydryl/thiol residues of non-heme proteins, lipid derivatives and can even be converted to nitrous gases (Pegg & Shahidi, 2000). As a result, only small residual nitrite levels (below 5 mg/kg) can be detected in the dry fermented sausages (Chapter 4.3.1 and Chapter 5.3.2.1). Consequently, only low amounts of both precursors are present. On the other hand, after 3 days of fermentation, the pH decreases from approximately 5.7 to values between 4.5 and 5.5. Due to this acidification, the nitrosation is slightly favoured. Nevertheless, subsequently, the sausages are dried and the a_w decreases from ca. 0.96 to values between 0.82 - 0.90, whereby the nitrosation is inhibited. Moreover, in contrast to the sterile liquid systems, dry fermented sausage may contain microflora which probably can degrade the formed NPIP again (Chapter 5.3.2.2).

In conclusion, the conversion to NPIP will be minute during the production of the sausages, since the preceding results demonstrate that the pH and a_w optima for NPIP formation can be avoided.

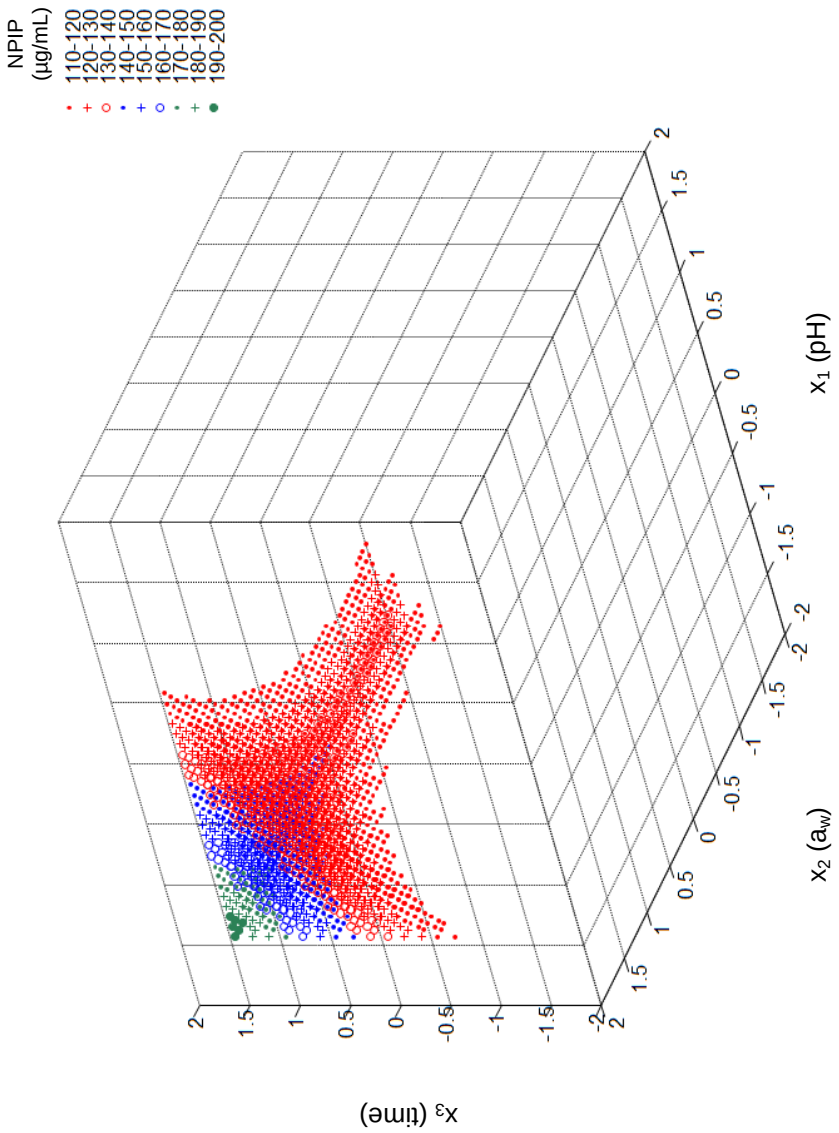


Figure 6.4 Predicted results for the grid points resulting in the highest NPIP concentrations.

6.4 CONCLUSIONS

At both pH levels (pH 4.0 and 5.0) the NPIP formation was reduced by addition of high amounts of NaCl. However, it was unclear to what extent these effects could be attributed to the higher ionic strength or the lower a_w. In contrast, the use of PEG in the system could, without introducing high salt concentrations, efficiently decrease the water activity similar to dry fermented sausages.

Three major conclusions could be drawn from the response surface plots, derived from the quadratic model based on the experimental design experiments applied on the PEG system. Firstly, in contrast with observations in dry fermented sausages (Chapter 5.3.2.2), the NPIP concentrations increased during incubation, which is probably related to the lack of microbial degradation in the sterile environment of the liquid system. Secondly, based on our results, the NPIP formation is maximal at pH 3.0. Thirdly, the NPIP formation can be inhibited by reducing the water activity.

Although the results obtained in the systems overestimate the formation in real dry fermented sausages, they contribute to a better understanding of the combined effect of pH and water activity on the NPIP formation during the production of dry fermented sausages. The model simulations clearly show that the pH and a_w significantly influence the formation of NPIP. In conclusion, as long as the water activity is low and decreasing, and there is no extreme acidification during the fermentation, it is not likely that the addition of sodium nitrite and PIP (from pepper) will form a major risk for the formation of carcinogenic NPIP in dry fermented sausages.

CHAPTER 7
RISK ASSESSMENT OF PIPERINE AND PIPERIDINE CONTAINING SPICES[6]

[6] This chapter was based on following paper:
De Mey, E., De Maere, H., Dewulf, L., Paelinck, H., Sajewicz, M., Fraeye, I., Kowalska, T. (2013). Assessment of the *N*-nitrosopiperidine formation risk from piperine and piperidine contained in spices used as meat product additives, European Food Research and Technology. DOI: 10.1007/s00217-013-2125-4

7.1 INTRODUCTION

Regarding the observations of Chapter 5, it can be concluded that NPIP contamination in dry fermented sausages can be caused by the addition of PIP to the meat batter. However, extreme high concentrations of PIP were required to form small amounts of NPIP in the end product. In addition, as discussed in Chapter 6, the pH and a_w conditions, related to the fermentation and ripening processes, were found to be inadequate to enhance the NPIP formation during the production of dry fermented sausages. Nevertheless, sporadically high levels of NPIP can be observed in commercial products (Chapter 3). As a proof of concept, the contribution of piperine and PIP containing spices to the NPIP formation is studied in this chapter.

PIP is a cyclic secondary amine, which can be considered as a parent molecular structure for many plant alkaloids, and piperine is the main pungent compound of pepper (*Piper nigrum*, Piperaceae). The chemical structure of both compounds is given in Figure 1.15. White pepper is produced from the seeds, i.e., the dried fruit without the pericarp. While black pepper is obtained by drying the whole unripe berry (Nair, 2004). Both pepper varieties are used for many culinary purposes. The pungency, and thus the quality, of pepper are related to the amount of piperine (Verzele *et al.*, 1989). However, this quality can be influenced by the hydrolysis of piperine, resulting in the cleavage of the PIP ring (Saikh *et al.*, 2006). As discussed in Chapter 1.4.2.1, the addition of spices to nitrite curing mixtures can contribute to the formation of *N*-nitrosamines during the storage. Therefore the use of such kind of premixes is strongly discouraged, although the direct relation between the concentration of piperine or PIP and the NPIP formation has not been established yet.

The aim of this study was threefold. Firstly, the methods for the determination of piperine and PIP in spices were developed, in order to assess the quality of the commercial pepper samples. Secondly, the piperine and PIP concentrations in a selection of culinary spices were measured. And finally, the NPIP formation was evaluated during the storage of certain spices in combination with the nitrite curing salt. In that way, possible risk of the NPIP formation was assessed, caused by piperine and PIP contained at different concentration levels in spices.

7.2 EXPERIMENTAL

7.2.1 SPICES AND NITRITE CURING MIXTURES

On the one hand, samples of different quality grades of white and black pepper (*P. nigrum*) were selected. *Inter alia*, they physically differed in particle diameter, namely cracked pepper with 1 - 2 mm particle diameter and pepper powder with a 0.3 mm average particle diameter. Hereby, the powder was either produced by fine grinding (further in the text referred to as

powder), or by industrial extracting and drying the spice (further referred to as extract). Besides the pepper samples, some other spices were also purchased, namely paprika (*Capsicum annuum*) and chili pepper (*Capsicum frutescens*), both belonging to the plant family of the nightshades (Solanaceae), and also allspice (*Pimenta dioica*, Myrtaceae) and nutmeg (*Myristica fragans*, Myristicaceae). The spice samples were purchased either from the local supermarket (the black pepper powder came from Delhaize, Brussels, Belgium), or directly from the two suppliers, nutmeg powder, white pepper powder and white pepper extract came from Raps (Kulmbach, Germany), the paprika, chili and allspice powder, and also the cracked white pepper and the Muntok white pepper originated from Rejo (Nazareth-Eke, Belgium).

In order to evaluate the risk of the NPIP formation, meat curing mixtures were prepared. These premixes consisted of 3 g of one of the above mentioned spices in 100 g nitrite curing salt (Rejo), which on its turn is composed of NaCl + 0.6% $NaNO_2$. These mixtures were vacuum packed and stored in the darkness at 25 °C. For tracing the NPIP formation, the mixtures were sampled (n = 3) after one day, one week, and two months storage period.

7.2.2 CHEMICALS AND STANDARDS

Hydrochloric acid (37% HCl), potassium hydroxide pellets (KOH), and antifoam silicone were all obtained from VWR International (West Chester, PA, USA). Methanol and DCM were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Water used in the experiment was de-ionized and double distilled in our laboratory by means of the Elix Advantage model Millipore system (manufactured in Molsheim, France).

The piperine (≥ 97%), PIP (≥ 99%), NPIP, and NDPA standards were purchased from Sigma-Aldrich (St Louis, MO, USA.). For the purpose of the HPLC analyses, piperine was dissolved in DCM at a concentration of 0.10 mg/mL, and PIP was dissolved in water at a concentration of 1.00 mg/mL. The NPIP standard was dissolved and diluted in DCM to obtain a concentration of 0.25 µg/mL. Quantification was carried out using NDPA as an IS at a concentration of 1 µg/mL DCM.

7.2.3 ACCELERATED SOLVENT EXTRACTION

Prior to the HPLC analysis of piperine and PIP, the ASE 200 model extractor (Dionex, Sunnyvale, CA, USA.) was used to carry out the accelerated solvent extraction on the spice samples. A 0.5-g portion of the dry spice was carefully weighed and placed in the stainless steel cell and underwent three consecutive extraction runs. A pressure of 100 atm was applied. The remaining working parameters were, as follows: the solvent (DCM or water) volume, 24 mL; static time, 15 min; number of extraction cycles within one extraction run, 2. Three 24-mL portions of extract obtained from a single

spice sample were merged in a 100-mL calibrated volumetric flask and filled up to 100 mL with DCM or water, respectively. These solutions were used for the chromatographic quantification of piperine and PIP in the investigated spices.

In order to optimize the extraction temperature for piperine with DCM as an extraction medium, the white pepper powder from Raps (containing a high level of piperine) was used as a model spice at six different working temperatures (50, 60, 65, 70, 75 and 80 °C). For the isolation of PIP, the temperature of hydroextraction was optimised and this time, the white pepper extract from Raps (containing a high level of PIP) was used as a model spice at six different working temperatures (40, 45, 50, 55, 60, and 65 °C).

7.2.4 RECOVERY STUDY

In order to evaluate the efficiency of the ASE procedure, a recovery study was performed. Therefore, the ground black pepper fruit was used as a model sample for the preparation of the blank plant matrix sample. In order to exhaustively extract piperine and PIP from this sample, the aforementioned ASE procedure with DCM and H_2O, respectively, as a solvent was repeated six consecutive times.

One series of the blank matrix samples was spiked with piperine (0.100, 0.200, 0.500 and 1.000 mg/g) and extracted with DCM. Another series of the blank matrix samples was spiked with PIP (0.100, 0.200, 0.500 and 1.000 mg/g) and extracted with H_2O. From each dried and spiked matrix, four 1-gram portions were weighed out and each portion underwent a single ASE extraction run. Then the four extracts obtained in parallel from the four equally spiked matrix samples were condensed in a flow of nitrogen at 40 °C to a volume of 1 mL each, with use of the TurboVap LV model evaporator (Zymark, Hopkinton, MA, USA.). These condensed solutions were then utilized for the chromatographic HPLC recovery studies and standard deviation was calculated from the recovery repetitions.

7.2.5 DETERMINATION OF PIPERINE AND PIP BY HPLC

The HPLC analyses were carried out using a Varian model 920 liquid chromatograph (Varian, Harbor City, CA, USA) equipped with a Varian 900-LC model autosampler, a gradient pump, a Varian model 330 DAD detector, a Varian 380-LC model ELSD detector, and the Galaxie software for data acquisition and processing. The analyses were carried out in the isocratic mode, using a Pursuit 5 C18 (5 µm particle size) column (250 mm × 4.6 mm i.d.; Varian). As mobile phase, methanol at a flow rate of 0.5 mL/min in the isocratic mode, was employed. The retention times (t_R) of piperine and PIP were equal to 5.9 and 10.9 min, respectively. Piperine, containing an aromatic chromophore, was detected by a diode array detector (DAD). For quantification, the peak heights at the wavelength of 343 nm (i.e., at the absorption maximum of piperine) were measured. The HPLC results valid for

PIP were derived from the evaporative light scattering detector (ELSD). The characteristics of the HPLC method are given in Table 7.1.

Table 7.1 Calibration curves obtained for piperine and PIP by HPLC, and the respective MDL and MQL values (n = 6).

Calibration curve	Piperine	PIP
y = ax + b	y = 554.296x–08.602	y = 40.145x–64.001
R^2	0.9565	0.9835
SD	46.747	70.070
MDL (mg/g)	0.028	0.58
MQL (mg/g)	0.084	1.74

7.2.6 DETERMINATION OF NPIP BY GC-TEA

The NPIP content was determined according to the *N*-nitrosamine method of Drabik-Markiewicz *et al.* (2011), as described in Chapter 3.2.3.3. The method was adapted for analyses of spices and nitrite curing mixtures. Therefore, 5-g samples were used instead of 50-g meat samples.

7.2.7 STATISTICAL ANALYSES

The results were expressed as the means ± standard deviation. The linear least-square regression was used to calculate the intercepts (a), slopes (b) and coefficients of determinations (R^2) of the respective calibration curves (Microsoft Office Excel 2007, Microsoft Corporation, Redmond, WA). Significant differences among the extraction temperatures were assessed with a one-way ANOVA (PASW Statistics 19.0.0, SPSS Inc.). Piperine and PIP contents of the spices and the accumulation of the NPIP content in the blends were also subjected to one-way ANOVAs. Tukey's honestly significant difference criterion ($p < 0.05$) was used to compare the means.

7.3 RESULTS AND DISCUSSION

7.3.1 EVALUATION OF THE ASE PROCEDURE

Most frequently, piperine and PIP are extracted from plant material by Soxhlet extraction. In this study, ASE was applied as an alternative extraction technique, in order to reduce the analysis time and the solvent consumption. As can be seen from Figure 7.1, the extraction yield of piperine (measured after a single extraction run) increased, when elevating the working temperature of the ASE extraction. At temperatures higher than 70 °C, no significant increase of the extraction yield could be observed, so it was decided to employ a temperature of 70 °C for the DCM extraction of piperine. Due to an excellent solubility of PIP and practical insolubility of piperine (and the other alkaloids) in water, a selective hydroextraction method for the isolation of PIP was elaborated. Complete extraction of PIP was observed over the whole range of the employed temperatures (results

not shown). For practical reasons, the temperature of 50 °C was chosen for the hydroextraction of PIP (as the lowest working temperature of the ASE 200 apparatus is set on 40 °C). Thus, the hydroextraction of PIP could be done at a temperature considerably lower than that employed for the extraction of PIP with DCM.

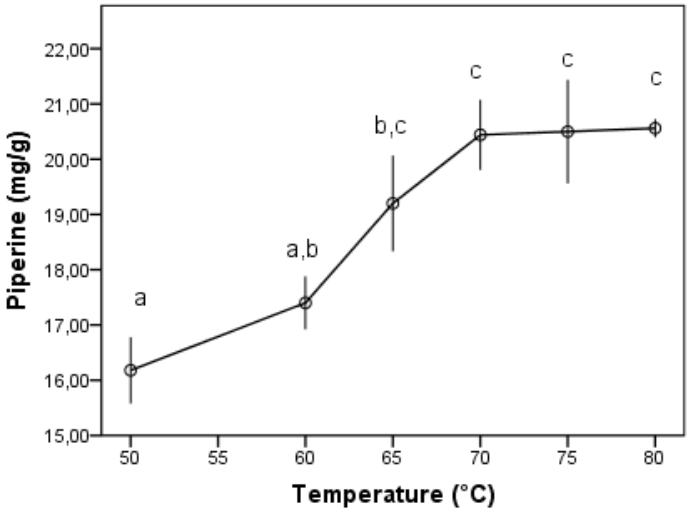


Figure 7.1 Effect of the extraction temperature on ASE extraction of piperine with DCM from the white pepper powder sample (n = 3). Data points with different letters are significantly different at $p < 0.05$ (Tukey's post hoc test).

In order to evaluate the accuracy and precision of both extraction procedures, recovery studies were performed as well. For that purpose, a blank plant matrix was prepared from the black pepper powder by thoroughly extracting piperine with DCM and PIP with water, using the ASE technique. After the six consecutive runs, neither of the two compounds could be chromatographically detected in the final extract. Hence, after drying the extracted plant material, it could be used as a blank plant matrix for the recovery study. The obtained results showed an excellent performance of the ASE extraction system and practically full recovery of the two compounds of interest from the spiked plant matrix (Table 7.2). Moreover, the recovery precision was confirmed by a good repeatability of the recovery results (RSD of ca. 2 - 3%). This successful outcome allowed using the calibration curves derived for the piperine and PIP standards from the HPLC quantification experiments (without a tedious spiking of the blank plant matrix, in order to prepare the plant-material-based calibration curves).

Table 7.2 Recovery (T%) and the repeatability (r) expressed as RSD (%) of piperine and PIP from 1 gram dry blank plant matrix derived from the ground black pepper fruit and spiked with four different concentrations of the test compounds (n = 4).

Compound	Addition level (mg/g)	Measured content (mg/g)	T (%)	RSD (%)
Piperine	0.100	0.098	98.4	2.80
	0.200	0.198	98.8	2.76
	0.500	0.496	99.2	2.89
	1.000	0.989	99.2	2.11
PIP	0.100	0.092	92.3	3.54
	0.200	0.189	94.5	3.11
	0.500	0.491	98.2	2.74
	1.000	0.985	98.5	2.79

7.3.2 PIPERINE AND PIPERIDINE CONTENT IN SPICES

In table 7.3, the results of the piperine and PIP analyses for the commercially available spices are given. In the white and black pepper samples (*P. nigrum*), a maximum of 21.12 mg/g piperine was measured, although in literature, the piperine contents usually show within the range of 30-80 mg/g (Schulz *et al.*, 2005; Liu *et al.*, 2013). Nevertheless, lower amounts have been reported as well, especially due to the ageing of the pepper samples (Verzele *et al.*, 1989). In the white pepper extract, a significantly lower amount of piperine was found. In the other investigated spices, only low amounts (± 2 mg/g) of piperine were detected, and no significant differences among these samples could be observed.

In the pepper samples (*P. nigrum*), the PIP content was in most cases a fraction of the piperine content, except for the sample of the white pepper extract in which the concentrations were almost equal. This industrial extract of white pepper contained the highest level of PIP among all tested spices, while its piperine content was rather low. Although no detailed information is available about the industrial production of the spice extract, it is clear that the composition of the white pepper extract is affected by the industrial extraction process. The piperine content apparently decreased, while the PIP content was remarkably higher in the white pepper extract than in the pulverized white pepper samples. Considering the changed ratio of the two alkaloids, it can be expected that piperine degrades during the production of the extract, e.g., under the influence of heat (Nisha *et al.*, 2009).

In paprika and chili PIP was not detected, while in allspice and nutmeg only traces were found. In the literature, no evidence could be found on meaningful amounts of PIP or PIP alkaloids in the spices from the families Solanaceae, Myrticaceae and Myristicaceae, so that our results remain in conformity with the literature data.

Table 7.3 Contents (mg/g) piperine and PIP in the spice samples (mean ± standard deviation; n = 6).

Spice sample	Piperine[1,2]	PIP[1,2]
White pepper (*P. nigrum*)		
powder (Raps)	20.65 ± 0.84[c,d]	3.70 ± 0.11[b]
cracked peppercorns (Rejo)	19.18 ± 0.66[c]	nd
powder, Muntok (Rejo)	20.80 ± 0.52[d]	5.83 ± 0.17[c]
extract (Raps)	12.12 ± 0.31[b]	11.42 ± 0.29[e]
Black pepper (*P. nigrum*)		
powder (Delhaize)	21.12 ± 0.75[d]	6.49 ± 0.21[d]
Paprika (*C. annuum*)		
powder (Rejo)	1.96 ± 0.06[a]	nd
Chili pepper (*C. frutescens*)		
powder (Rejo)	1.97 ± 0.08[a]	nd
Allspice (*P. dioica*)		
powder (Rejo)	2.11 ± 0.08[a]	*0.66 ± 0.03*[a]
Nutmeg (*M. fragrans*)		
powder (Raps)	2.17 ± 0.06[a]	*0.69 ± 0.03*[a]

[1]nd: not detected, values below the MDL, [2]values estimated below the MQL are printed in italics. Different letters in the same column indicate significant differences at $p < 0.05$ (Tukey's post hoc test)

7.3.3 Evaluation of the NPIP formation in nitrite curing mixtures

Four spice samples, including white pepper powder and nutmeg powder which were used in the dry fermented sausage preparations (as described in Chapter 4), were then tested for the risk of the NPIP formation. As can be seen from Table 7.4, NPIP could initially not be detected in the analysed spices, which suggests their safe use in food products. One day after the preparation of the nitrite curing salt blended with these spices, no detectable amount of NPIP was found in any of them. After one week, however, the curing mixture containing the white pepper powder showed a NPIP contamination, which significantly increased after the two months storage period. In agreement with the results of earlier studies (Sen *et al.*, 1974b; Gough & Goodhead, 1975), the presence of the secondary amines (such as PIP), can cause a risk of *N*-nitrosamines formation. For this reason, the FDA strongly discourages combination of nitrites and nitrates with spices (USDA, 2012). In contrast, no advices for the use of premixes was found in European legislation. However, during the two months storage period of this particular spice premix, only a small fraction of the initial level of PIP was converted to NPIP in the presence of an excessive amount of $NaNO_2$ (0.6 % in the nitrite curing salt). When in one kg of meat batter an amount of ca. 28 g of this spice premix is added instead of the regular nitrite curing salt (see Chapter 4.2.3), the introduced NPIP concentration would be below MDL of 0.9 µg/kg DM (see Chapter 5.2.2). This means that, for the preparation of dry fermented sausages, the use of spice premixes with comparable levels of NPIP contamination cannot explain the occurrence of detectable amounts

of NPIP in the sausages. During this storage experiment, it was confirmed that NPIP can be formed under dry circumstances which occur in these kind of premixes, however the reaction rate seemed to be very low. Possibly, only after a very long storage period (> 2 months), premixes may contain NPIP levels, high enough to be considered as a meaningful NPIP contamination source in dry fermented sausages. In fact, in a preliminary study (unpublished data), commercial spice mixtures, of which the exact composition and age was not known, were found to be contaminated with much higher concentrations of NPIP. Probably, the NPIP formation was accelerated by incorrect storage conditions (temperature abuse, higher relative humidity, prolonged storage,…). Moreover, these kind of blends are available on the market and thus can be the source of quantifiable NPIP contamination in dry fermented sausages.

Table 7.4 NPIP contents (ng/g) in the spice samples and during storage of some nitrite curing mixtures (mean ± standard deviation; n = 3).

	Spice	Curing mixture		
Spice sample		1 day	1 week	2 months
White pepper (*P. nigrum*)				
powder (Raps)	nd	nd	3.28 ± 0.19[a]	9.80 ± 0.41[b]
extract (Raps)	nd	nd	nd	nd
Paprika (*C. annuum*)				
powder (Rejo)	nd	nd	nd	nd
Nutmeg (*M. fragrans*)				
powder (Raps)	nd	nd	nd	nd

different letters in the same row indicate significant differences at $p < 0.05$.

Although NPIP was formed in the curing mixture containing the white pepper powder, all the other curing mixtures remained free from NPIP. As anticipated, no NPIP was formed in the mixtures prepared with nutmeg and paprika, due to very low concentrations of piperine and PIP in these spices. However, based on an elevated PIP level, NPIP formation was expected in the mixture produced with use of the white pepper extract. Probably in that case, the absence of NPIP formation can be linked with an altered composition of white pepper in the course of the industrial extraction process (Wang, Jiang, & Li, 2013). It is known from the literature that many spices contain antioxidants, such as polyphenols and the nitrite scavenging compounds (Baliga, *et al.*, 2003; Tsai *et al.*, 2007), and the activity of these compounds is higher in the extracts than in the unprocessed spices (Yanishlieva *et al.*, 2006). In that case, antioxidants and nitrite scavenging compounds can probably inhibit the *N*-nitrosamines formation (Li *et al.*, 2013). Finally, it has to be emphasized that no information is available on the production process of the white pepper extract and on the polyphenolic composition contained therein, and further research is needed to reveal the inhibitory effects of this extract.

7.4 CONCLUSIONS

Rapid and accurate accelerated solvent extraction procedures were developed to facilitate quantification of piperine and PIP in spices by means of HPLC. For the determination of piperine, the ASE extraction was employed with DCM (70 °C) as an extraction solvent while PIP was isolated from the spice matrix by application of a hydroextraction at 50 °C. For both alkaloids, good recovery was obtained by means of ASE.

The piperine contents in the pepper samples were considerably higher than the trace levels in the spices belonging to the botanical plant families of Solanaceae, Myrtaceae, and Myristicaceae. The PIP content in the extract of the white pepper powder was considerably higher than that found in the pulverized samples. As predicted by the low piperine and PIP levels, nitrite curing mixtures prepared with nutmeg and paprika did not show any NPIP contamination. Against our expectations, the use of the white pepper extract, containing a high piperine concentration, did not induce NPIP formation. In contrast, the use of the white pepper powder in the curing mixtures caused an increase of the NPIP content from not detected to 9.80 ± 0.41 ng/g after the two month storage period. Nevertheless, after a storage period of two months, these concentrations were too low to explain the occurrence of NPIP in dry fermented sausages. In future work, it can be investigated if alternative storage conditions (temperature abuse, higher relative humidity, prolonged storage,…) of these kind of premixes can lead to an increased formation of NPIP. In conclusion, high amounts of piperine and PIP in the spices do not necessarily result in the NPIP formation, which depends on the total composition and the industrial processing of the spices and extracts.

GENERAL CONCLUSIONS

Since a half century research has been done in order to decrease the *N*-nitrosamines content in meat products. In this period, the most urgent problems were solved and important precautions (e.g., the restriction of added sodium nitrite and the use of antioxidants like sodium ascorbate) were introduced to prevent severe *N*-nitrosamine formation. Although the total *N*-nitrosamine content of meat products is nowadays strongly reduced, sporadically *N*-nitrosamines, even in high amounts, may occur.

To avoid contamination of meat products with carcinogenic *N*-nitrosamines, this doctoral work was carried out to gain additional insight in the occurrence and the formation of *N*-nitrosamines, especially NPIP. In the next paragraphs, the main conclusions of this doctoral study, their relevance to meat safety and the suggestions for future research are discussed.

To realize one of the objectives, i.e., the investigation of the occasional relationship between biogenic amines, nitrosating agent and *N*-nitrosamines, it was required to rely on accurate analytical methods to determine possible precursors (e.g., biogenic amines, nitrite and nitrate) as well as the *N*-nitrosamines in dry fermented sausages. On the one hand, the analytical method for the determination of volatile *N*-nitrosamines, i.e., NDMA, NDEA, NDBA, NPYR, NPIP and NMOR, could be adopted from the preceding PhD study of Drabik-Markiewicz and the determination of the nitrite and nitrate content could easily be based on methods found in literature. On the other hand, the development of an analytical method to determine the biogenic amines, i.e., TRYP, PHE, CAD, PUT, HIS, SER, and TYR, and the natural polyamines, i.e., SPM and SPD, in dry fermented sausages required the development of a robust method (Chapter 2). Hereby, good sensitivity was obtained by a derivatisation with dabsyl chloride and the matrix interferences were reduced by a subsequent solid phase extraction. Repeatability, accuracy and reproducibility demonstrated that the method was suitable for the determination of biogenic amines in dry fermented sausages.

Since the beginning of the worldwide *N*-nitrosamine research, a positive trend could be observed in the reduction of volatile *N*-nitrosamines. Nevertheless, to date, still reports are being published wherein the *N*-nitrosamine contamination of several types of meat products, including dry fermented sausages, is mentioned. In this work, a survey of dry fermented sausages, commercially available on the Belgian market (Chapter 3), revealed that the concentrations of the investigated *N*-nitrosamines were generally low. Nevertheless, NMOR and NPIP were found in more than one quarter of the samples. Hereby, the sporadic occurrence of unexplained high concentrations of NPIP was specifically noticed. Despite the application of exploratory data analyses, i.e. HCA and PCA, no direct relationship was

found between the accumulation of biogenic amines, residual nitrite and nitrate concentrations and the occurrence of the *N*-nitrosamines. This can be attributed to the great variety in formulations of the commercial dry fermented sausages and the lack of information of the exact process conditions. Therefore, it was impossible to determine the causes of the *N*-nitrosamine contamination in commercial meat products.

In distinction to many other studies, the role of precursors was evaluated in a real meat model, since it gives the opportunity to provide additional information concerning the *N*-nitrosamine formation in a meat matrix containing many interfering compounds. In this study, the use of a well-controlled, but realistic dry fermented sausage model, was chosen. The developed model (Chapter 4), based on a North European style dry fermented sausage preparation, could be produced under strictly controlled conditions in the technical plant of the Research Group for Technology and Quality of Animal Products. Hereby, the end product showed comparable physical (pH and a_w) and chemical characteristics (salt, moisture, protein and free fat content) as the investigated commercial sausages. In addition, the sausages, made according this production process, can be considered safe since the accumulation of biogenic amines could be inhibited by the use of sodium nitrite and no distinct *N*-nitrosamine formation was observed (Chapter 5). Therefore, the production of this type of sausage can be used as a representative model for the study of *N*-nitrosamine formation.

In the case commercial samples were contaminated with *N*-nitrosamines (Chapter 3), NPIP was most frequently detected (28%). Therefore, with the objective to gain additional insight for the production of safe dry fermented sausages, the second part of this work was focused on the NPIP formation.

On the one hand, the biogenic amine CAD is postulated in literature as a precursor of NPIP. However the artificial addition of a large amount of CAD in the meat batter, simulating the use of putrefactive meat for the preparation of sausages, could not invoke the formation of NPIP or other *N*-nitrosamines (Chapter 5). In this way, it was demonstrated that the accumulation of biogenic amines, at least with regard to CAD, does not mean a risk for the formation of *N*-nitrosamines during the production of dry fermented sausages under GMP conditions. On the other hand, the addition of high amounts of the direct precursor PIP could induce NPIP formation during the production of the dry fermented sausage model, although the detected amounts were influenced by factors like the use of nitrite and ascorbate and dependent of the time during production. In fact, the highest NPIP levels were observed in the freshly stuffed sausages, even before the fermentation process. Subsequently, during the fermentation and ripening, the NPIP levels were decreasing, which is probably attributed to the microbial degradation by the microflora present in the sausages. Therefore, the effect of nitrite as precursor and ascorbate as inhibitor of the NPIP formation could only be observed in the freshly stuffed sausages and not at the end of production.

From the results obtained of the experiments carried out in the dry fermented sausage model, it can be concluded that *N*-nitrosamines are not formed during the production of dry fermented sausages under GMP conditions. In the PhD work of Drabik-Markiewicz (2010), the necessity of a high heating temperature in the formation of several volatile *N*-nitrosamines was demonstrated. However the production of dry fermented sausage does not include a heating step. Therefore, in this work, the combination of the reaction parameters pH and a_w, which play an important role during the production of dry fermented sausages, were for the first time evaluated concerning their effect on the NPIP formation. Since it was impossible to make sausages with fixed pH and a_w values over a broad range, protein-based liquid systems were used to study the combined effect of pH and a_w on the formation of NPIP (Chapter 6). In the first liquid system, NaCl was used to reduce a_w levels, which clearly inhibited NPIP formation. However, to achieve comparable a_w levels as can be found in dry sausages, high NaCl concentrations (up to 30%) were needed in the liquid system. In that case, it was unclear to what extent inhibition of NPIP formation could be attributed to the higher ionic strength or the lower a_w. Moreover the applied high NaCl concentrations did not reflect percentages occurring in dry fermented sausage, and therefore an alternative liquid system was also applied. In the second system PEG was used to reduce the a_w, while the pH was altered by the addition of lactic acid. By means of the experimental design CCD, it was possible to test several variables simultaneously. Subsequently, the results obtained by RSM, implicated that as long as the a_w is low (<0.95) and the acidification during the fermentation is within the normal range (4.5-5.5), it is not likely that NPIP will be formed. At least when nitrite is added in amounts permitted by legislation and PIP is introduced in the meat formulation in levels naturally occurring in pepper, no detectable amounts of NPIP will occur in dry fermented sausages.

Since it is observed that CAD is not a precursor of NPIP, it can be assumed that the deamination and cyclization of CAD to form PIP will not take place during the production of dry fermented sausages. In consequence, PIP, recognized as a possible precursor of NPIP, must originate from other sources than the biogenic amine accumulation in the meat. Therefore, as a proof of concept, it was studied if the occurrence of NPIP in dry fermented sausages could be related to the use of piperine and PIP containing spices (Chapter 7). On the one hand, in black and white pepper (*P. nigrum*) samples, both compounds were found in considerably higher amounts. However, the PIP concentrations in dry fermented sausages, introduced by the addition of these spices were not high enough to result in detectable amounts of NPIP. On the other hand, NPIP was formed in a mixture of white pepper with nitrite curing salt during the storage period of two months. Although, the NPIP content of the mixture was too low to explain the NPIP levels found in the commercial dry fermented sausages, former analyses of commercial spice blends demonstrated the occurrence of extreme high amounts of NPIP. Probably the formation in these kind of mixtures was induced by longer storage period or incorrect storage conditions.

In this work, it can be concluded that the occurrence of quantifiable amounts of NPIP in dry fermented sausages is not related to the presence of secondary amines (CAD and PIP) derived from the meat itself or introduced in the meat batter by use of PIP containing spices like pepper. In addition, it was proven that the changes within the pH and a_w range, which occur during the production of dry fermented sausages, do not form an additional risk in the formation of NPIP. These results, combined with the findings of the work of Drabik-Markiewicz (2010), indicate that meat products, produced under normal process conditions (heating to maximum 160°C, acidification above pH 4.5 and drying below a_w 0.95), are safe concerning the formation of *N*-nitrosamines, at least with regard to NPIP.

When NPIP is detected in commercial dry fermented sausages, more likely the contamination originates from external sources. Although NPIP formation may occur in seasonings, containing piperine and PIP rich spices, the results of the storage experiment of spices blended with nitrite curing salt (Chapter 7) were not convincing enough to demonstrate this hypothesis. Most probably, the storage conditions were not critical enough. This hypothesis can be investigated more thoroughly in future research. Hereby, alternative storage conditions (e.g., temperature abuse, higher relative humidity, prolonged storage) can be introduced in order to reveal an increased formation of NPIP in these mixtures. Thereby, it should be noted that despite the fact that premixes of spices and the additives nitrite and nitrate are already discouraged, still spices and herbs, rich in nitrate, are commonly combined with pepper in seasoning premixes. Therefore, it is also advised to include these types of premixes in the future research project. Besides obtaining insight in possible contamination sources, it is also interesting to study the *N*-nitrosamine degradation, which was observed in the dry fermented sausage model (Chapter 5). As discussed earlier, it is believed that the degradation is attributed to microbial activity. Therefore, in addition to this PhD work, which was focused on the interaction of precursors and physical and chemical reaction parameters, the presence and growth of microorganisms in dry fermented sausages, possessing (i) nitrosating enzymes and (ii) metabolic activity towards *N*-nitrosamines can be investigated. In this way, an additional strategy can be developed to assure safe meat products.

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